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Research Article

A complex of Shc and Ran-GTPase localises to the cell nucleus

R. George[a], H.-L. Chan[b], Z. Ahmed[a], K. M. Suen[a], C. N. Stevens[a], J. A. Levitt[c], K. Suhling[c], J. Timms[b] and J. E. Ladbury[a,*]

[a] Department of Biochemistry and Molecular Biology, University College London, Gower Street, London, WC1E 6BT (UK), Fax: 44 (0)20 7679 7193, e-mail: j.ladbury@biochem.ucl.ac.uk
[b] Cancer Proteomics Laboratory, EGA Institute for Women's Health, University College London, Cruciform Building 1.1, Gower Street, WC1E 6BT (UK)
[c] Department of Physics, Kings College London, Strand, London WC2 2LS (UK)

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Abstract. The three isoforms of the adaptor protein Shc play diverse roles in cell signalling. For example, the observation of p46 Shc in the nuclei of hepatocellular carcinoma cells suggests a function quite distinct from the better characterised cytoplasmic role. Ligands responsible for the transport of various Shc isoforms into organelles such as the nucleus have yet to be reported. To identify such ligands a far western approach was used to determine the p52 Shc interactome. The Ran-GTPase nuclear transport protein was identified and found to bind to p52 Shc *in vitro* with low micromolar affinity. Co-immunoprecipitation, pull down and fluorescence lifetime imaging microscopy experiments in stable cells confirmed cellular interaction and nuclear localisation. The nuclear transport factor protein NTF2, which functions in cohort with Ran, was shown to form a complex with both RAN and Shc, suggesting a mechanism for Shc entry into the nucleus as part of a tertiary complex.

Keywords. Nuclear localisation, protein complex, FLIM, nuclear transport, proteomics.

Introduction

The role of the Shc family of adaptor proteins in binding to receptor tyrosine kinases [1, 2, 3] and transducing signals to the MAP kinase pathway via Grb2-Sos is well documented [4, 5]. This family of proteins contains three isoforms. The p66 isoform, the largest of the Shc proteins, has an N-terminal CH2 domain which is not expressed as part of the other two isoforms. While the p52 and p46 isoforms are ubiquitously expressed, p66 Shc is found mainly in neuronal cells and functions in oxidative stress management [6]. All three isoforms have a common domain architecture consisting of an N-terminal phosphotyrosine binding (PTB) domain, a central collagen homology 1 (CH1) domain and a C-terminal SH2 domain. The p52 Shc also possesses an unstructured N-terminal tail of 44 amino acids that is absent from the p46 isoform. The structure and binding of the PTB domain has been characterised [7, 8] and it is known to be required for the recruitment of Shc to the juxtamembrane region of receptor tyrosine kinases [9, 10]. Three tyrosine residues within the CH1 domain can be phosphorylated and are involved in the recruitment of other adaptor proteins such as Grb2 [11] and Gads [12]. The CH1 domain also contains several proline-rich motifs which potentially provide binding sites for SH3 domain-containing proteins; however, only three ligands have been

* Corresponding author.

reported to date [13]. A limited number of binding partners have been reported for the SH2 domain. Direct interactions have been demonstrated with the T cell receptor [14], CEACAM1 [15] and cadherin [16]. Additional binding partners such as Lck [17], PP2A [18], estrogen receptor α [19], mPAL [20], PTP-PEST [21] and PTPε [22] appear to interact with the PTB or SH2 domains of Shc without themselves having to be tyrosine phosphorylated. Recently we reported that p52 Shc has an inherent phosphorylation-dependent gating mechanism where the phosphotyrosine binding site of the SH2 domain only becomes available when the CH1 domain is tyrosine phosphorylated [23]. It is thought that once localised to the juxtamembrane region of receptors via its PTB domain, the SH2 domain of Shc is able to interact directly with the receptor or facilitate the recruitment of other proteins into early signalling complexes [24] prior to MAP kinase activation. Despite reported data on the isolated PTB and SH2 domains, the structure of intact Shc remains to be determined; however, circular dichroism spectroscopic data suggest that the functional form of p52 Shc adopts a largely unfolded conformation [23].

The multiple domain architecture of the Shc family of proteins provides the opportunity for simultaneous binding of a variety of different proteins and is suggestive of a multi-functional cellular role. Recently the molecular chaperone Pin1 was shown to interact with the p66 isoform of Shc. This interaction is required for entry into the mitochondria during oxidative stress, resulting in the onset of apoptosis [25, 26]. The p46 Shc isoform has been shown to migrate to the mitochondria [27] and, in certain cancer cells, to the nucleus [28, 29]. The mechanism of Shc transport into the nucleus and its role within the nuclear environment is unknown. The Shc isoforms do not have a discernable nuclear localisation sequence and, hence, must enter the nucleus via an independent mechanism. This capacity of Shc to function both in the cytoplasm and in organelles such as the nucleus suggests that it is able to interact with transport proteins required for localisation; none of which have been experimentally confirmed to date.

In this study a proteomics-based analysis has been adopted to determine the p52 Shc interactome using far western methodology [30]. This approach allows for multiple binding partners to be probed simultaneously using the purified protein of interest. In our case, whole cell lysates, separated by 2D elecrophoresis and transferred to a nitrocellulose membrane, were probed with purified p52 Shc. Interacting proteins were then determined by performing a western blot with anti-Shc antibody and subsequently identified by mass spectroscopic analysis. The protein Ran was discovered as a novel binding partner. Recently it has been shown that Ran, in complex with the nuclear translocation factor 2 (NTF2), can import protein into the nucleus [31]. Ran is a Ras-like small GTPase that plays key roles in nuclear export and import and in mitotic spindle formation (see [32, 33] for reviews). The expression of Ran is known to be up-regulated in many cancer cell types [34, 35]. Interestingly, silencing of Ran using RNAi technology was shown to promote apoptosis of transformed cells, a feature that did not occur in untransformed cells [35]. Hence, inhibiting the function of Ran has been proposed as a useful strategy in the fight against malignant growth. We show that Ran binds to p52 Shc with low micromolar affinity. Analysis of immunoprecipitation, pulldown, fluorescent cell images and fluorescence lifetime imaging microscopy (FLIM) data indicate that Ran and Shc can interact directly in the cell through the SH2 domain of Shc, pointing to the strong possibility of Ran-mediated Shc transport into the nucleus. This view is enhanced by the observation that NTF2 can form complexes with both Shc and Ran. Thus, it appears that Shc is transported into the nucleus as part of a tertiary complex with Ran and NTF2 [31]. This provides an explanation as to why, in transformed cells, the presence of Shc in the nuclei is concomitant with the observed elevated levels of Ran.

Methods and materials

Protein expression and purification of p52Shc. p52Shc was expressed and purified as described previously [23]. Briefly, Shc was expressed as a His-tagged construct using Rosetta 2 strain of *E. coli* at 30°C. After lysis and sonication Shc was purified using metal affinity purification, anion exchange, and finally size exclusion chromatography. Shc was found to be greater than 98% pure as assessed by SDS PAGE.

Expression and purification of Ran. The plasmid pKSW305 coding for His-tagged wt canine *ran* (a kind gift from Dr. Karsten Weiss, University of California, Berkeley) was transformed into Rosetta2 strain of *E.coli.* A single colony was picked from a freshly transformed plate and used to inoculate 100 ml LB which was grown at 37°C overnight. 10 ml of the resulting culture was used to inoculate 1 L of LB which was grown at 30°C until the OD 600 nm = 0.8, at which point 5% ethanol was added to the media and the temperature lowered to 25°C. When the OD 600 nm reached 1.0 Ran synthesis was induced by addition of 0.5 mM IPTG. The culture was allowed to grow for a

further three hours, after which the cells were harvested by centrifugation and stored at -20°C. Four litres of Ran pellet were lysed by sonication in the lysis buffer (50 mM Tris HCl pH 8.0, 200 mM NaCl, 10 % glycerol) in the presence of protease inhibitors. Insoluble material was removed by centrifugation (20 000 g at 4°C for 45 min). The soluble fraction was applied to a metal affinity column containing Talon (Clontec) beads. The beads were washed thoroughly with buffer and then 10 mM imidazole. Ran was eluted with a 100 mM imidazole wash in 50 mM Tris HCl pH 8.0, 100 mM NaCl. The sample was concentrated to 5 ml and applied to a superdex S200 gel filtration column in buffer containing 50 mM Tris HCl pH 8.0, 200 mM NaCl, 1 mM DTT.

Expression and purification of CypA. The plasmid pETCypA (a kind gift from Dr. M. Walkinshaw, University of Edinburgh) coding for His-tagged CypA was transformed into BL21(DE3) for expression. A single colony was used to transform 100 ml of LB which was grown overnight at 37°C. 4 x 1 L of LB were inoculated with 10 ml of this overnight culture and were allowed to grow at 37°C until the OD 600 = 0.5, at which point CypA expression was induced by the addition of 1 mM IPTG. Cultures were allowed to grow for a further four hours, after which time the cells were harvested by centrifugation and stored at -20°C until required. CypA was purified in exactly the same way as Ran (see above) and was greater than 95 % pure as judged by SDS-PAGE.

Expression and purification of Grb2. Grb2 was expressed as a His-tagged fusion protein in BL21(DE3) *E. coli.* Three litres of cell pellet were used to prepare Grb2. Grb2 was purified using the same method as CypA and Ran (see above). Grb2 was assessed to be greater than 98 % pure by SDS PAGE.

Purification of Shc SH2 and PTB domain GST fusion proteins. The SH2 and PTB domains of Shc were purified as GST fusion proteins from BL21(DE3) *E.coli.* Large 1 L cultures were grown from LB inoculated with 10 ml of overnight culture at 37°C. IPTG was added to the cultures when the OD 600 = 0.5, after which growth was continued for a further four hours. Harvested cells were lysed in sonication buffer (see above) and the soluble fraction added to a GST Bind Resin (Novagen). The protein was eluted with lysis buffer containing 10mM reduced glutathione. The GST fusion proteins were concentrated to 5 ml and applied to a S200 Sephadex size-exclusion column in buffer containing 50 mM Tris HCL pH 8.0, 200 mM NaCl, 1 mM DTT.

Tissue culture. HEK293T cells were cultured in DMEM media supplemented with 10 % FBS and appropriate dilutions of antibiotic/antimycotic reagent (Invitrogen). Cells were transfected with plasmids expressing Ran-GFP and Shc-RFP using lipofectamine with the procedure outlined by the manufacturer. A stable cell line expressing Ran-GFP was created and used for pulldown and immunoprecipitation experiments (see below). In order to detect an interaction between Ran and Shc, a plasmid expressing Shc fused to RFP was transfected into the stable cell line expressing Ran-GFP. These transiently transfected cells were then used for FLIM analysis (see below).

Pulldown and immunoprecipitation experiments. In order to determine which domains of Shc bound to Ran, pulldown experiments were performed using GST fused SH2 and PTB domains of p52 Shc. Equal quantities of each domain and GST alone, bound to GST bind resin (Novagen), were incubated overnight at 4 °C with lysates from two preparations of stable HEK293T cells expressing Ran-GFP. The first of these had cells in the basal state and the second contained cells which were quenched after 15 min stimulation with 5 % FBS. After incubation the beads were washed with 3 x 1 ml of lysis buffer, resuspended in 20 µl of SDS PAGE loading buffer and boiled. Western blotting using anti-GFP antibody was used to determine the presence of Ran-GFP. Because of the alternate start site in the gene of p52 Shc, expression of this in cells can lead to p46 expression. This could lead to a low level of background expression of p46 Shc. Our *in vitro* data demonstrate a clear capability for p52 Shc to interact with Ran, giving confidence that the cell based assays reveal the effects of p52 Shc binding.

Jurkat cell lysis and CyDye-labelling. Jurkat cells were cultured in RPMI (Cambrex) media with 10 % FBS and appropriate dilutions of antibiotic/antimycotic solution (Invitrogen) at 37°C and 5 % CO_2. Cells were washed twice in PBS, drained well and lysed in 2D lysis buffer (4 % (w/v) CHAPS, 8 M urea, 10 mM Tris-HCl, pH 8.3, 1 mM EDTA) without reducing agent. Samples were homogenized by passage through a 25-gauge needle (six times) and insoluble material removed by centrifugation (13 000 rpm/10 min/4°C). Protein concentration was determined using BioRad Protein Assay Reagent. To facilitate alignment of 2D gel-separated proteins with far western blots, extracts were labelled with NHS-Cy5 at 4 pmol of dye/µg of protein for 30 min on ice. Reactions were quenched with a 20-fold molar excess of lysine to dye, prior to addition of DTT to 65 mM. Volumes were adjusted to

350 μL with 2D lysis buffer plus DTT and carrier ampholines (pH 3–10) added to 2% (v/v).

Two-dimensional gel electrophoresis. For 2-DE, 18 cm, non-linear pH 3–10 immobilised pH gradient (IPG) strips (GE Healthcare) were rehydrated with Cy-labelled samples in the dark at room temperature overnight. Isoelectric focusing was performed on a Multiphor II (GE Healthcare) for 80 kVh at 20°C. Strips were equilibrated for 10 min in 50 mM Tris-HCl pH 6.8, 6 M urea, 30% (v/v) glycerol and 2% (w/v) SDS containing 65 mM DTT and then for 10 min in the same buffer containing 240 mM iodoacetamide (IAM). Equilibrated IPG strips were then transferred onto 1.5 mm thick 18 X 20 cm 12% polyacrylamide gels cast between low-fluorescence glass plates. Strips were overlaid with 0.5% (w/v) low-melting point agarose in running buffer with bromophenol blue. Gels were run in Protean II tanks (BioRad) at 10 mA per gel at 10°C until the dye front had run off the bottom. All steps were carried out in a dedicated clean room.

Detection of Proteins. 2D gels were scanned between low-fluorescence glass plates using a Typhoon 9400 multicolour fluorescence scanner and ImageQuant software (both GE Healthcare). Gels were post-stained with Colloidal Coomassie Blue G-250 (CCB) to visualise gel separated proteins. Briefly, gels were fixed in 35% (v/v) ethanol, 2% (v/v) phosphoric acid for >3 h, washed 3 x 30 min in dd H_2O and incubated in 34% (v/v) methanol, 17% (w/v) ammonium sulphate, 3% (v/v) phosphoric acid for 1 h, prior to addition of 0.5 g/l Coomassie G-250 (Merck) and left to stain for 2–3 days. No destaining step was required. Stained gels were scanned on a Typhoon™ 9400 scanner using the red laser and no emission filters. The same Cy5-labelled protein extracts on replica 2D gels were electroblotted onto Immobilon P membrane (Millipore) and subjected to far western blotting using recombinant Shc or Grb2 (see below). Briefly, membranes were blocked for 1 h with 5% (w/v) BSA in Tris-buffered saline (50 mM Tris pH 8, 150 mM NaCl) plus 0.1% Tween-20 (TBS-T) and then probed with purified Shc and Grb2, and then with anti-Shc monoclonal antibody (BD signalling) or anti-Grb2 monoclonal antibody (BD signalling). Membranes were then incubated for ≥ 1 h in primary antibody in TBS-T, washed in TBS-T (3×10 min) and probed with the appropriate horseradish peroxidase-coupled secondary antibody. After further washes in TBS-T, immunoprobed proteins were visualised using enhanced chemiluminescence (PerkinElmer Life Sciences Inc). Films were scanned on a BioRad GS-800 densitometer and images aligned with gel and membrane CyDye and CCB images using Adobe Photoshop.

Protein Identification by LC-MS/MS. Spots of interest were excised from CCB stained gels and subjected to in-gel digestion with trypsin, as previously described [36]. Peptide extracts were then vacuum-dried and resuspended in 6 μl dd H_2O containing 0.1% formic acid. LC-MS/MS was performed by injecting 5 μl of digested peptides onto a reversed phase capillary column (PepMap 75 μm x 150 mm, LC Packings) using a nanoflow HPLC system (Ultimate, Dionex) connected on-line to an ESI Q-TOF I mass spectrometer (Waters, UK). The flow rate was 300 nl/min and separation was performed by gradient elution from 5–50% solution B (80% (v/v) acetonitrile, 0.1% formic acid) in 60 min followed by an isocratic step at 100% solution B for 10 min. Balance solution A was 0.1% formic acid. Data-dependent acquisition was used with MS scans set every second (m/z 350–1500) and MS/MS performed on automatically selected peptide ions, also for 1 second (m/z 50–2000, continuum mode), using the function switching in MassLynx V4.0 software. Raw MS/MS data were smoothed (Savitzky Golay, two channels twice) and centroided (at 80%) and peaks lists generated using MassLynx software. Peak lists were submitted for database searching using Mascot (V2.1.06). Searches were performed against the Human IPI database (release version 3.29; May 2007; 68 161 sequence entries). Parameters for protein searches were: enzyme (trypsin, porcine); miscleavages (2); charge of ions (+2 and +3); mass tolerance of precursor peptide ion (100 ppm) and mass tolerance for MS/MS fragment ions (0.8 mmu). Carbamidomethylation of cysteines was considered as a fixed modification, whilst oxidation of methionine, pyro-glutamic acid and N-acetylation were considered as variable modifications. Positive protein identifications were called when at least two peptide sequences matched an entry and Mascot scores were above the significance threshold value at $P = 0.05$.

Isothermal Titration Calorimetry (ITC). ITC measurements were recorded using a VP-ITC manufactured by MicroCal (Northampton, MA, USA) at 15°C. Shc was present in the calorimeter cell and Ran, CypA and Grb2 were loaded into the syringe. A binding isotherm was generated using twenty fifteen-microliter injections. All isotherms were fitted to a one-site binding model using least squares fitting analysis (Origin 5.0). Heats of dilution were subtracted from the isotherms before fitting.

Table 1. Thermodynamic parameters for the binding of Ran, CypA and Grb2 to p52 Shc at 15°C

Protein	KD(μM)	ΔH (kJ/mol)	TΔS (kJ/mol)	ΔG (kJ/mol)
Ran	0.68	–17.89	16.11	–34.00
CypA	0.76	–17.51	16.23	–33.74
Grb2	No binding	–	–	–

FLIM Analysis. Experiments were performed using an inverted confocal microscope (Leica TCS SP2) which was adapted for Time-Correlated Single-Photon Counting (TCSPC) FLIM with a Becker & Hickl SPC 830 card using 64 or 256 time channels in a 3 GHz, Pentium IV, 1GB RAM computer running Windows XP. The fluorescence decays were fitted with a single exponential decay model using Becker & Hickl's SPCImage software v2.8.3, and the GFP fluorescence lifetimes displayed in a false colour map.

Results and discussion

Far western analysis of Shc interacting proteins. In order to determine the p52 Shc interactome far western analysis was carried out using Jurkat whole cell lysates separated by 2D gel electrophoresis. Four gels were run in total. The far western procedure was adopted for two of the gels using either purified p52 Shc or Grb2 (as a control protein). One gel was stained with CCB and the other used in a western blot probed with anti-Shc antibody to determine any background signal due to non-specific binding. Figure 1 shows the results of the far westerns, the control blot and the CCB stained gel. The far western blot using p52 Shc (Fig. 1A) shows that Shc is able to bind to a large array of proteins from the Jurkat whole cell lysate. Approximately 150 interactions were counted in total. None of the observed interactions were due to non-specific binding of the Shc antibody, as the appropriate control immunoblot shows only three spots, which are likely to be differentially phosphorylated forms of p52 Shc (see Fig. 1B). The pattern of the interactions obtained on the control blot using Grb2 was very different to that of p52 Shc (Fig. 1C), confirming that the identified interacting proteins were unique. Repeating the Shc far western gave a very similar pattern of interactions (data not shown). Spots were picked from a CCB stained gel using the Cy5 signal from the labelled lysates to aid in the alignment (Fig. 1D). Excised protein spots were digested with tryspin then subjected to LC-MS/MS-based analysis to determine their identities (see Materials and methods). Out of the ~150 putative interactions, 45 proteins were identified with high confidence representing 37 unique gene products; these are listed in Supplementary Materials and annotated on the far western blot in Figure 2. The identified proteins could be grouped into five categories, comprising cytoskeletal proteins, nuclear proteins, molecular chaperones, proteasome machinery and metabolic enzymes. Encouragingly, the proteins tubulin alpha-3 (vimentin), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and DnaJ, identified in an independent study of complexes involving Shc in macrophages [37], were also found in this study.

The direct interaction of p52 Shc and Ran-GTPase. The analysis above identified the nuclear transport protein Ran as a binding partner for Shc. In order to validate direct binding of these, we expressed and purified both proteins from a bacterial host and determined the binding characteristics using ITC. Figure 3A shows the binding isotherm generated from the Ran titration. From the resulting fit, Ran was found to bind to p52 Shc with a K_D of 0.7 μM and with a stoichiometry of 1. The thermodynamic parameters determined from the interaction are given in Table 1. To validate the far westerns, further titrations with other putative binding partners for Shc were also carried out. For example, binding of the peptidyl-prolyl cis-trans isomerase, CypA was detected with similar affinity to that obtained for the Ran (see Table 1). As a further control for the binding of Ran we performed ITC experiments with Grb2 to determine binding to p52 Shc. As expected, no binding was observed to the non-phosphorylated form of p52 Shc (see Fig. 3B; [23]).

Immunoprecipitation, pulldown and FLIM analysis confirm binding of Ran to Shc in cells. In order to confirm if Ran could interact with Shc in cells, the *ran* gene was PCR amplified from the bacterial vector (see Methods and materials) and cloned into a mammalian expression vector that would produce a C-terminal GFP fusion upon expression in mammalian cell lines. This construct was transfected into HEK293T cells and a stable cell line produced. The interaction of endogenous Shc with Ran-GFP was determined by immunoprecipitation and immunoblotting experiments. These experiments clearly showed that the Shc isoforms and Ran-GFP can form a complex, both in the basal state and when cells have been stimulated

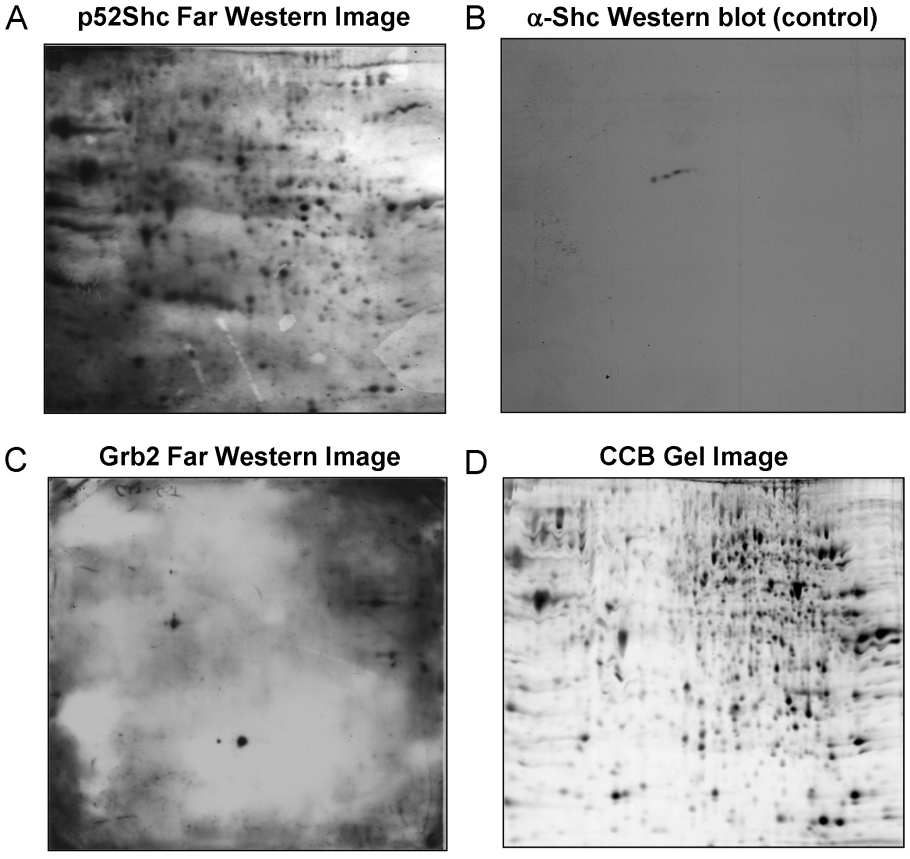

Figure 1. Far western analysis of Jurkat whole cell lysate. (*A*) Far western blot of Jurkat whole cell lysate showing putative p52 Shc-interacting proteins. (*B*) Western blot of Jurkat whole cell lysate probed with anti-Shc antibody. (*C*) Far western control experiment showing Grb2-interacting proteins. (*D*) Gel image of Jurkat whole cell lysate stained with colloidal Coomassie Blue (CCB).

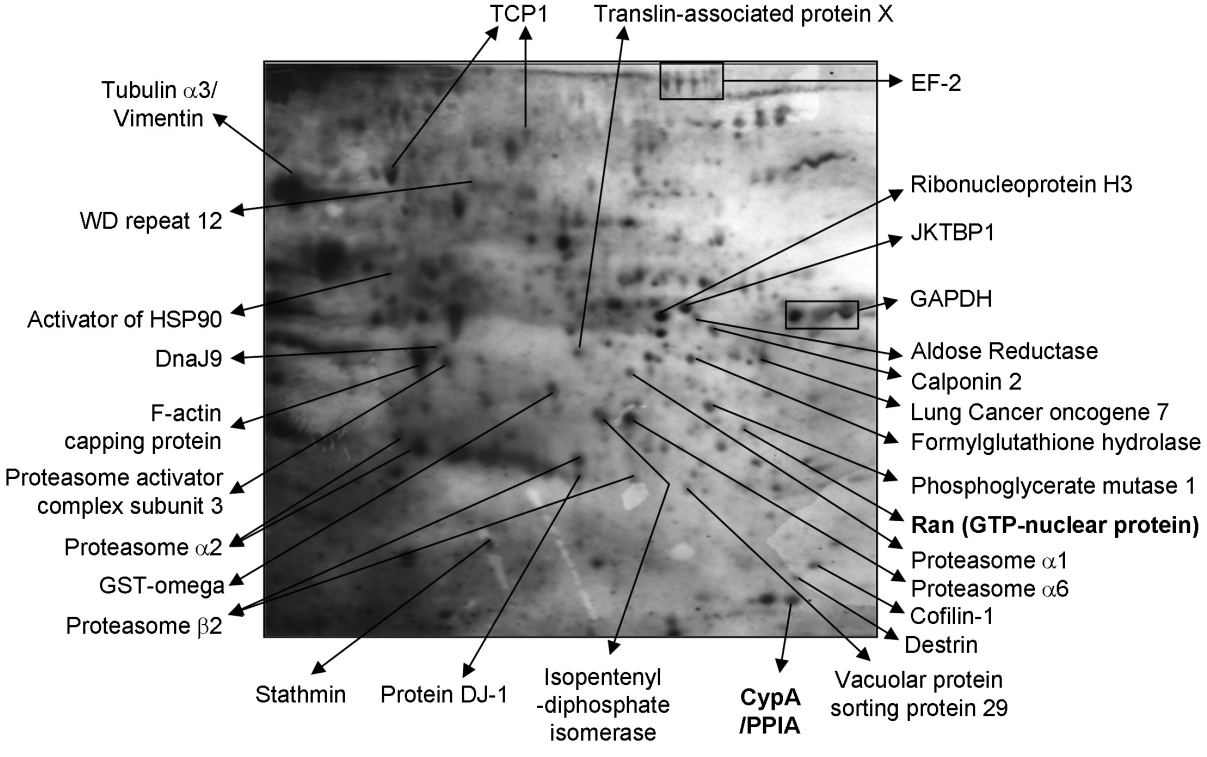

Figure 2. p52 Shc far western blot showing positions and identities of interacting proteins (see Table 1 – Supplementary Materials).

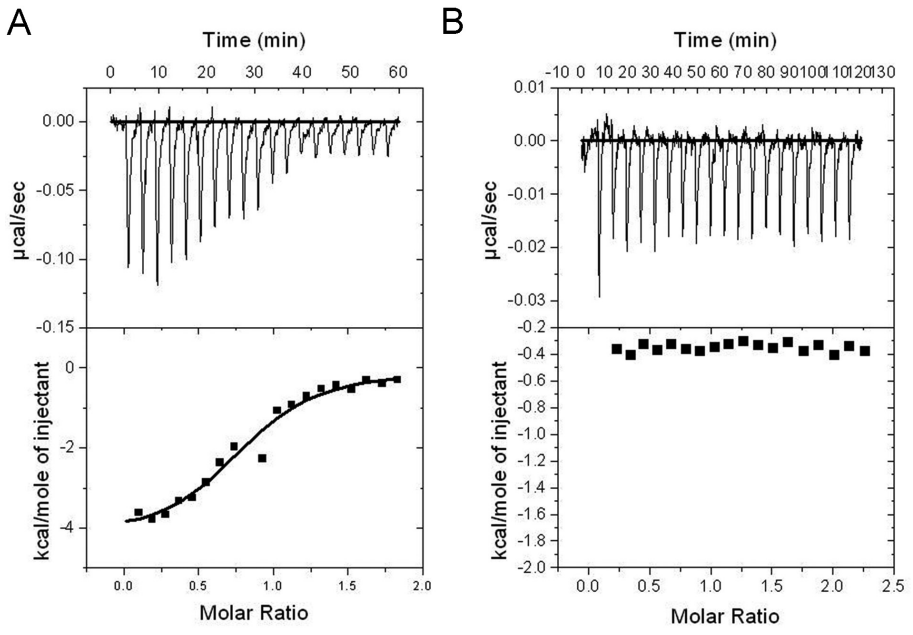

Figure 3. ITC analysis of p52 Shc binding to Ran and Grb2. Upper panels show raw data for the binding isotherm generated upon titration of Shc with (*A*) Ran and (*B*) Grb2. Lower panel (*A*) shows the integrated heats from the raw data with the independently determined heats of dilution subtracted. Lower panel (*B*) shows the integrated heats from the raw data. Titrations were carried out at 15°C with Shc present in the calorimeter cell. The Ran isotherm was fitted to a one site binding model using Origin 5.0.

with 5% FBS for 15 min (see Figs. 4A and B). Interestingly, the Shc IP showed an increase in the amount of coprecipitated Ran upon stimulation. Also, as indicated with the GFP immunoprecipitation (Fig. 4A), only the p52 and p46 isoforms but not the p66 isoform of Shc could coprecipitate with Ran. To determine which domains of Shc were responsible for the interaction, pulldown experiments from whole cell lysates were carried out using the SH2 domain of Shc fused to GST. Analysis of the proteins bound to the domain by western blotting with anti-GFP antibody showed that the interaction of isoforms of Shc with Ran was likely to be via the SH2 domain (Fig. 4C). Since Ran is not known to undergo tyrosine phosphorylation, this interaction is likely to be via a similar mode as seen for other interactions with the PTB and SH2 domains with non-phosphorylated proteins (see above).

Immunoprecipitation confirmed that the presence of a GFP tag did not interfere with Ran binding to Shc. In order to determine if this interaction was direct we carried out FLIM analysis by detection of FRET between Ran-GFP and Shc-RFP (derived from a transiently transfected plasmid). Firstly, we examined images for cells individually expressing p46 and p52 isoforms of Shc-RFP and Ran-GFP (see Fig. 4D). The localisation of Ran and Shc are distinct in these cells. Ran is expressed mainly in the nucleus whilst the Shc isoforms are, as expected, predominantly located in the cytoplasm. Figure 4E shows the fluorescent cell images of co-transfected cells in the basal state. The images clearly show that a significant proportion of the exogenously expressed Shc-RFP is localised to the nucleus (second and third panels from top), an effect that can be attributed to the presence of co-expressed Ran-GFP. It should be noted that there is a greater than 10-fold increase in Ran expression compared to untransfected cells. Therefore the Ran levels here closely resemble those found in cancerous cells [35]. Analysis of the fluorescence lifetime of the Ran-GFP in the basal state (fourth panel from top) shows a strongly reduced fluorescence lifetime of the GFP due to FRET between GFP and RFP, indicating a direct interaction (bottom panel; histogram is left-shifted to shorter lifetimes). GFP has a typical fluorescence lifetime of approximately 2 ns (marked on panel) [38]. Taken as a whole, these data strongly indicate a Ran-mediated import mechanism of Shc into the nucleus. It is unclear from our data which Shc isoform is present in the nucleus. The p52 Shc-RFP construct used in this study can also generate the p46 Shc-RFP isoform, due to the presence of an alternate start site within the gene. As immunoprecipitation experiments indicate that both the p46 and p52 isoforms interact with Ran, it is possible that both isoforms may gain entry to the nucleus (see Fig. 4A). This conclusion is further enhanced by the fact that the interaction between Shc and Ran is mediated by the SH2 domain, which is common to both isoforms. However, reports exist where only the p46 isoform of Shc has been located in the nucleus of liver and gastric cancer tumour cells, whereas no studies exist that show the nuclear localisation of either isoform in untransformed cells [28, 29]. It is becoming increasingly clear in the literature that cancer cells have elevated levels of Ran [35], which has been suggested

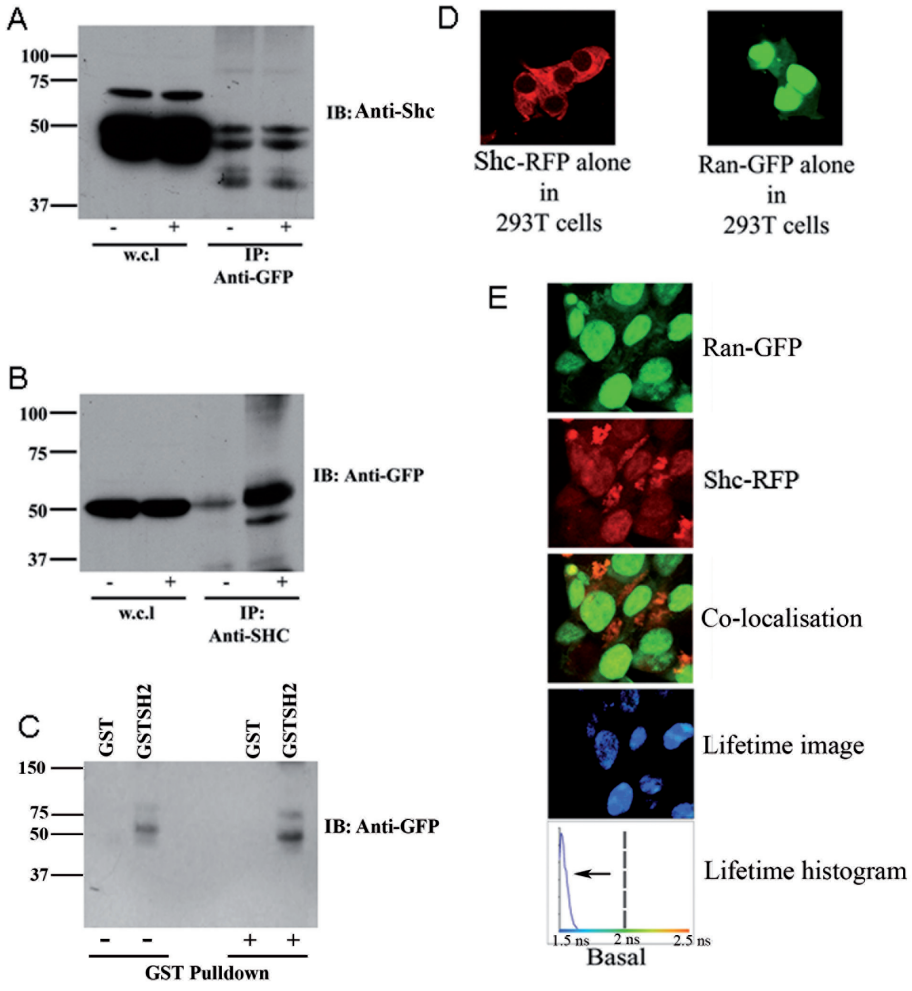

Figure 4. Immunoprecipitation, pulldown and FLIM analysis show that Shc interacts with Ran in cells. (*A*) Immunoprecipitation of Ran-GFP: HEK293T cells stably over-expressing Ran-GFP were serum starved overnight and then lysed (-) or stimulated with FBS and then lysed (+). 2 μg of whole cell lysate (w. c. l) and 2.5 μg of anti-GFP antibody were used for each immunoprecipitation. The resulting immunoprecipitates together with 100 ng of whole cell lysate (w. c. l) were immunoblotted with anti-Shc antibody. (*B*) Immunoprecipitation of Shc: Shc was immunoprecipitated from cell lysates as above and immunoblotted with anti-GFP antibody. (*C*) Pulldown analysis to determine the SH2 domain interaction of Shc with Ran: GST-SH2 domain of Shc expressed and purified from bacteria immobilised on agarose beads was incubated with cell-lysates as above along with GST alone control. The precipitants were immunoblotted with anti-GFP antibody (*D*) Confocal images of HEK239T cells expressing either Shc-RFP or Ran-GFP (*E*) Detecting the interaction of Shc-RFP and Ran-GFP by FLIM analysis: Stable HEK-293T cells expressing GFP-tagged Ran were transiently transfected with RFP-tagged Shc. At 24 h post-transfection, cells were seeded on to coverslips and allowed to grow for a further 48 h. Cells were serum-starved overnight, fixed and analysed by confocal microscopy. Co-localization of Shc–RFP with Ran-GFP was acquired. The lifetime measurements were made over a 300 s acquisition period, the fluorescence intensity image and the lifetime histogram mapped to an arbitrary colour scale from blue to red. The fluorescence lifetime distribution for all pixels in the field of view is provided in the bottom panels with the same colour scale

to lead to defects in the nucleo-cytoplasmic transport of proteins and mislocalization of oncogenes and tumour suppressors [39]. With this in mind, it is possible that our observation of Shc in the nucleus may be a consequence of overexpression of Ran similar to the effect in cancer cells and may also account for the significant localisation of Shc in the cytosol of the cotransfected cells (see Fig. 4E). However, the interactome data indicates a number of other nuclear localised proteins that may interact with Shc (See Table published online at SpringerLink), further pointing to a possible role for Shc in the nuclei of untransformed cells.

NTF2 can form complexes with both Ran and Shc. The role of Ran in cytoplasmic-nuclear transport has been reviewed extensively [32, 33]. Ran has been shown to cycle between the cytoplasm and the nucleus bound to either GDP or GTP. Ran is concentrated in the nucleus and maintained in the GTP bound form by the Ran-GEF, RCC1. Cytoplasmic Ran is in the GDP bound form due to the presence of the Ran-GAP

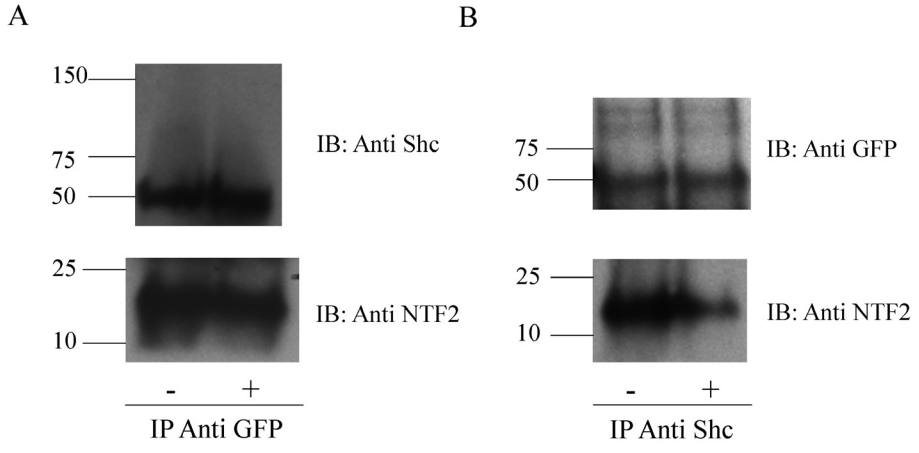

Figure 5. NTF2 forms complexes with Shc and Ran. Immunoprecipitation of Ran-GFP or Shc in both basal and stimulated cells results in the detection of NTF2. (*A*) Anti-GFP immunoprecipitate probed with Shc and NTF2 antibodies or (*B*) anti-Shc immunoprecipitate probed with NTF2 antibody.

which stimulates Ran GTPase activity. Recycling back into the nucleus is dependent on Ran forming a complex with nuclear transport factor 2 (NTF2) [40]. Recently, the Ran-NTF2 complex was shown to bind the ubiquitously expressed actin capping protein, CapG, and transport it into the nucleus [31]. CapG nuclear import was shown to be dependent on a stable Ran-NTF2 complex. This complex, therefore, may well function in the translocation of Shc. To assess whether the transport of Shc into the nucleus could be accompanied by NFT2 we investigated the potential for the formation of a tertiary complex between Shc, Ran and NTF2. Immunoprecipitation of these proteins was carried out in both basal cells and in cells stimulated with 5% FBS (see Fig. 5A). In both immunoprecipitation experiments NTF2 was detected, indicating that both Shc and Ran can interact with NTF2.

Conclusion

The herein adopted proteomics-based approach reveals proteins which form the interactome of p52 Shc. These data confirm that Shc is likely to be involved in a wide range of cellular functions. Here, we demonstrate that, in quiescent and stimulated cells which over-express Ran, Shc makes a direct interaction with Ran. This interaction is nuclear localised in transformed cells in the basal state and forms a tertiary complex involving both Ran and NTF2. Thus, over-expression of Ran in cells seems to provide a vehicle for nuclear entry. The role adopted once localised, however, is yet to be recognised. The nuclear localisation of the Shc p46 isoform in hepatocellular carcinoma cells suggests a potential oncogenic function which might identify it as a viable target for pharmaceutical intervention.

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