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The Ran binding protein RanBPM interacts with Axl and Sky receptor tyrosine kinases

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Abstract

Axl belongs to a particular subfamily of transmembrane receptor tyrosine kinases, the biological ligand for which is the growth/survival factor Gas6. However, little is known about the molecular mechanisms for Axl activation and signal transduction. We have previously identified a novel interaction between the intracellular domain of Axl and Ran binding protein in microtubule organising centre (RanBPM). In the present study, we investigated further the nature of the RanBPM interaction with Axl. A wide distribution of RanBPM mRNA expression in human tissues and various human cancer cell lines was detected. The strength of interaction of both proteins in yeast was comparable to that with the other Axl-binding proteins phosphatidylinositol 3-kinase and Grb2. A truncated version of RanBPM with the SPRY-LisH domain region omitted failed to interact with Axl in yeast. RanBPM was also found to interact in yeast with the Axl homologue, Sky/Tyro3. The interaction between Axl intracellular domain and RanBPM was reproduced in coimmunoprecipitation experiments in both cell-free and mammalian cell systems. Furthermore, coimmunoprecipitation revealed endogenous Axl and RanBPM to interact in several mammalian cell lines in a constitutive manner. Stimulation of COS cells with Gas6 caused increased Axl tyrosine phosphorylation although appeared not to influence the RanBPM–Axl association. In conclusion, we have identified and characterised a novel interaction between RanBPM and the related receptor tyrosine kinases, Axl and Sky. This novel insight into the signalling interactions of Axl and Sky may shed further light on their suspected roles in tumourigenesis, inflammation as well as other cell proliferative diseases.

Keywords: Axl; Receptor tyrosine kinase; Ran binding protein; Gas6; Signal transduction

1. Introduction

Axl is a member of a distinct subfamily of transmembrane receptor tyrosine kinases (RTK), also including Sky and c-Mer, all of which share significant identity in amino acid sequence and extracellular domain compositions (Manning, Whyte, Martinez, Hunter, & Sudarsanam, 2002). These receptors contain two N-terminal immunoglobulin-like domains followed by two fibronectin type III motifs, forming an extracellular structure resembling neural cell adhesion molecules (Stitt et al., 1995). The biological ligand for all three receptors is the protein product of growth

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arrest specific gene 6 (Gas6), a vitamin K-dependent protein homologous to anticoagulant protein S (Manfioletti, Brancolini, Avanzi, & Schneider, 1993). Gas6 shows highest affinity for Axl and both receptor and ligand have been implicated in cellular growth and transformation processes. Axl overexpression or activation is strongly associated with several types of tumours. Axl was first identified as a transforming gene from NIH3T3 cells (O'Bryan et al., 1991), and has since been observed to be aberrantly expressed in myeloproliferative disorders (Neubauer et al., 1997), breast cancer (Berclaz et al., 2001), prostatic carcinoma (Jacob et al., 1999) and thyroid carcinoma (Ito et al., 2002). In addition, Axl activation by Gas6 has been shown to stimulate cell survival, proliferation (Goruppi, Ruaro, & Schneider, 1996) and migration (Fridell, Villa, Attar, & Liu, 1998).

The signal transduction pathways activated by the Gas6–Axl interaction remain to be fully elucidated. The lipid kinase phosphatidylinositol 3-kinase (PI3K) and its substrate, the serine/threonine kinase Akt appear to occupy pivotal positions in the survival pathway (Lee, Wen, Varnum, & Hung, 2002). In support of this, direct protein–protein interactions have been revealed between the Axl intracellular domain (Axl-IC) and PI3K, phospholipase C-γ and Grb2 (Braunger et al., 1997). We have in addition identified Nck2, SOCS-1, C1-TEN and RanBPM as novel intracellular Axl-binding proteins (Hafizi, Alindri, Karlsson, & Dahlback, 2002). Ran Binding Protein in Microtubule organising centre (RanBPM/RanBP9) was first identified as one of a number of proteins interacting with the small GTPase Ran and localised to both the nucleus and cytoplasm (Nakamura et al., 1998a). However, membrane-localised interactions with RanBPM have since been reported for a number of different proteins, including MET RTK (Wang, Li, Messing, & Wu, 2002a), steroid receptors (Rao et al., 2002), the neurotrophin receptor (Bai, Chen, & Huang, 2003) and β_2 integrin (Denti et al., 2004). RanBPM contains a highly conserved SPRY (SPla and the RYanodine receptor) domain followed by a LisH-CTLH motif combination (Lissencephaly type-1-like homology and C-terminal to LisH), the former having been shown to interact directly with MET (Wang et al., 2002a).

In the present study, we characterise further the novel molecular interaction between Axl and RanBPM that we previously identified through yeast two-hybrid screening. We have verified the interaction further in yeast, where we also found Sky/Tyro3 to interact with RanBPM. The strength of the RanBPM interaction with Axl was determined relative to that of other Axl binding proteins. Furthermore, we have localised the interaction to the SPRY domain of RanBPM and carried out an analysis of expression of RanBPM in a variety of human tissues and cancer cells. In addition, we also demonstrated interaction between the endogenously expressed Axl and RanBPM proteins in mammalian cells as well as examining their response to Gas6, the biological ligand for Axl. Our findings indicate that RanBPM is a novel interacting protein for Axl RTK and may therefore play a role in Gas6/Axl signal transduction.

2. Materials and methods

2.1. Northern blot analysis

Human multiple tissue northern (MTN) and cancer cell line blots (BD Clontech) were probed for expression of RanBPM mRNA. A 1.4 kb fragment was isolated from the RanBPM cloned yeast cDNA by cleavage of two *BsaHI* sites and used to produce a ^{32}P-labelled probe as previously described (Hafizi et al., 2002). The cDNA sequence for human β-actin was used as a control probe. Radiolabelling with [^{32}P]dCTP was achieved through the Rediprime II DNA labelling system (Amersham). Hybridisation was carried out overnight at 42 °C in ULTRAhyb hybridisation buffer (Ambion), followed by high stringency washes. Membranes were then exposed to a PhosphorImager screen at least overnight prior to visualisation.

2.2. Plasmid construction

All reagents for use in yeast two-hybrid experiments were purchased from BD Clontech (Matchmaker System 3). For confirmatory experiments, yeast cells were transformed with plasmids encoding proteins of interest as fusions to either GAL4 DNA binding domain (BD; pGBKT7) or activation domain (AD; pACT2). The known specific association of murine p53-BD (pGBKT7-53) and SV40 large T antigen-AD (in pTD1-1) was used for positive control yeast two-hybrid interactions. As a negative control,

pGBKT7-Lam was used instead of pGBKT7-53, encoding lamin C-BD protein, which is known not to interact with large T antigen. The entire cytoplasmic domain of human Sky (Sky-IC, amino acids 452-890; GenBank accession no. AAH51756) was also prepared fused to GAL4 BD for studying against RanBPM-AD.

For coimmunoprecipitation experiments, the yeast plasmids encoding Axl-IC and RanBPM were used in T7 promoter-driven in vitro translation reactions, producing proteins lacking the GAL4 domains but possessing N-terminal Myc and HA tags, respectively. For this, RanBPM was prepared in pGADT7 plasmid by direct transfer of the original yeast two-hybrid cDNA sequence from pACT2 vector, using compatible *SfiI* and *XhoI* restriction enzyme sites.

For domain analysis, a truncated form of RanBPM (tRanBPM) was constructed by removal of a fragment from the RanBPM cDNA sequence through restriction enzyme digestion of two natural *EcoRI* sites, followed by religation. This yielded a protein product lacking 179 amino acids that encoded the entire SPRY and LisH domains. All cloning procedures and cDNA constructs were verified by restriction digestion analysis and DNA sequencing (Big Dye, Applied Biosystems).

2.3. Analysis of Axl– and Sky–RanBPM interactions in yeast

As previously described, the entire cytoplasmic domain of human Axl (Axl-IC, amino acids 473-894; GenBank accession no. NM_021913; Fig. 2A) was used as bait in a high stringency GAL4-based yeast two-hybrid screen (Hafizi et al., 2002). Axl-IC was fused to the DNA-binding domain (DNA-BD) of yeast GAL4 and an N-terminal c-Myc epitope tag. This was coexpressed in yeast with the products of a human heart cDNA library, which were fusions with the GAL4 activation domain (AD) and contained N-terminal haemagglutinin (HA) epitope tags. One of the positive yeast two-hybrid colonies that showed activation of all reporter genes, *HIS3*, *ADE2*, *lacZ* and *MEL1* coexpressed Axl-IC and RanBPM/RanBP9 (NCBI accession no. NM_005493). The Axl binding protein was a fusion of yeast GAL4 activation domain (AD) and amino acids 136–729 of full length RanBPM (Genbank accession no. BAB62525; Fig. 2B), i.e. the remainder of the protein after an initial stretch of N-terminal glutamine and proline repeats.

The positive yeast two-hybrid colony housing RanBPM was further analysed for expression of both bait and partner fusion proteins. The soluble yeast cell extract was analysed by 10% SDS-PAGE and western blotting as previously described, using anti-Myc and -HA antibodies (Santa Cruz Biotechnology) to detect bait and partner proteins, respectively, in the same culture (Hafizi et al., 2002).

The following plasmid constructs were transformed into yeast cells: Axl-IC-BD, tRanBPM-AD, Sky-IC-BD, RanBPM-AD, p53-BD and large T-AD. All transformed yeast cells were selected based on growth on appropriate selection medium, with cells housing both BD and AD plasmids able to survive on minimal synthetic dropout (SDO) plates lacking both Leu and Trp. Survival ability of test transformants was then compared by streaking onto YPDA nutrient plates as well as on quadruple dropout (QDO) plates (-Ade, -His, -Leu, -Trp) containing X-α-Gal (20 μg/ml), which turns colonies with α-galactosidase activity blue. Yeast plates were incubated at 30 °C for at least 3 days until growth was visibly apparent.

2.4. α-Galactosidase activity assay

The relative strength of interaction of RanBPM with Axl was analysed in a sensitive quantitative α-galactosidase (α-Gal) assay by comparing with other selected Axl binding partners that we have previously identified. This assay is based on expression of the *MEL1* reporter gene in the Matchmaker yeast two hybrid system (BD Clontech), where α-Gal activity secreted into the extracellular medium is measured (Aho, Arffman, Pummi, & Uitto, 1997). RanBPM–Axl and tRanBPM–Axl cultures were compared to yeast cultures containing Axl-IC coexpressed alongside PI3K p85 subunit or Grb2. Lamin C-large T antigen coexpression in yeast, showing no interaction was used as a negative control. These were cultured in liquid SDO medium for double transformants (-Leu, -Trp) at 30 °C overnight. Cells were pelleted by centrifugation and α-Gal catalytic activity in supernatants was monitored in a 96 well plate format by measuring the rate of hydrolysis of the chromogenic substrate *p*-nitrophenyl-α-D-galactoside (PNP-α-Gal; Sigma). The amount of the reaction product *p*-nitrophenol generated after a certain incubation period was measured spectrophotometrically at 410 nm. α-Gal

activity [milliunits/(ml × cell)] was calculated using the following formula:

$$\frac{\mathrm{Abs}_{410} V_f 1000}{\varepsilon b t V_i \mathrm{Abs}_{600}}$$

where t is the elapsed time (min) of incubation, V_f the final volume of assay (200 μl), V_i the volume of culture medium supernatant added (16 μl), Abs_{600} the optical density of overnight culture and ε is the molar absorption of *p*-nitrophenol, with a 1 cm light path (b). Abs_{600} was used to normalise the Abs_{410} of different media samples to the number of cells in each culture. Three separate colony cultures for each test construct were assayed in triplicate. Data underwent a one-way analysis of variance followed by a Bonferroni *t*-test for comparison between individual treatments. A *P* value of less than 0.05 was considered statistically significant.

2.5. Axl and RanBPM coimmunoprecipitation

The Axl-IC and RanBPM sequences from the yeast two-hybrid screen were also synthesised without GAL4 fusions for both cell-free and mammalian cell-based coimmunoprecipitation experiments. In the yeast vectors pGBKT7 and pGADT7 housing Axl-IC and RanBPM, respectively, a T7 RNA polymerase promoter lies between the GAL4 domain cDNA sequence and the sequence of interest. A linear DNA fragment incorporating the essential sequences was first isolated from the plasmid by digestion of two different restriction enzymes, followed by linked in vitro transcription and translation reactions previously described (PROTEINscript II, Ambion) (Hafizi et al., 2002). Emergent proteins were radiolabelled with [^{35}S]methionine and [^{35}S]cysteine, and contained either an N-terminal c-Myc epitope tag (Axl-IC) or HA tag (RanBPM). Directly after in vitro translation, 10 μl of reaction products were mixed together in a reaction tube and incubated at room temperature for 1 h. Samples were precleared by 1 h incubation in 10 μl of protein G sepharose beads (Amersham Biosciences), followed by centrifugation. A 1 μg of precipitating antibody (against either c-Myc or HA tag) was added to the relevant tubes and incubated at room temperature for 1 h. 10 μl of protein G sepharose beads was then added and the mixture further incubated on a rotator at room temperature for 1 h. After incubation, the immune complexes were subjected to five rounds of washing with ice-cold PBS and centrifugation at 5200 × *g* for 10 s. Finally, the supernatant was removed entirely and pelleted beads resuspended and boiled in 25 μl of 3× SDS loading buffer containing 1 mM DTT. Samples were subjected to 10% SDS-PAGE, after which the gel was fixed in 45% methanol/10% acetic acid, then dried and exposed to a PhosphorImager screen (Molecular Dynamics) at least overnight.

The mammalian COS cell line was also used for coimmunoprecipitation experiments with transfected RanBPM as the cells express endogenous Axl. Cells were transiently transfected with Myc-tagged RanBPM cDNA using expression vector pCMV-Myc as previously described (Hafizi et al., 2002). Axl protein was immunoprecipitated from lysates using goat polyclonal anti-Axl antibody (R&D Systems), followed by western blotting for Myc and Axl itself.

2.6. Coimmunoprecipitation of endogenous RanBPM and Axl in mammalian cell lines

Four different mammalian cell lines were analysed for coimmunoprecipitation of both endogenously expressed RanBPM and Axl: COS, the colorectal adenocarcinoma cell line SW480, the cervical cancer cell line HeLa S3 (all from ATCC) and the endothelial/epithelial hybrid cell line EAhy 926 (gift of Dr. Cora-Jean Edgell, University of North Carolina, USA) (Edgell, McDonald, & Graham, 1983). Confluent cells in 90 mm dishes were serum starved by overnight incubation in DMEM containing only 0.1% BSA. After this period, the medium was replaced with fresh DMEM containing no additives and incubated for a further 1 h prior to stimulation with recombinant human Gas6 (500 ng/ml) for 45 min. To stop the experiment, cells were rinsed with ice-cold PBS, then lysed in ice-cold lysis buffer composed of 1% NP-40, 1% sodium deoxycholate, 5 mM EDTA, 1 mM EGTA in PBS supplemented with a protease inhibitor cocktail (Sigma) and the tyrosine phosphatase inhibitor Na_3VO_4 (0.2 mM). Lysates were disrupted by running through a syringe needle and clarified by centrifugation at 14,000 rpm for 1 h at 4 °C. The soluble lysate supernatant was precleared by 1 h preincubation with protein G-sepharose beads (Amersham Pharmacia Biotech) and an irrelevant goat polyclonal antibody. Then, precleared cell

lysates, containing equal amounts of total protein (1 mg), were incubated first for 1 h with 2 μg goat polyclonal anti-Axl antiserum (C-20, Santa Cruz) then with 20 μl added protein G-sepharose, rotating overnight at 4 °C. After this period, immune complexes were washed five times with ice-cold lysis buffer, then solubilised in SDS loading buffer and analysed by 10% SDS-PAGE and western blotting as previously described (Hafizi et al., 2002). Membranes were first probed with a rabbit polyclonal anti-human RanBPM antibody (gift of Dr. Takeharu Nishimoto, Kyushu, Japan). Blots were developed using horseradish peroxidase-conjugate secondary antibodies (Dako, Glostrup, Denmark) followed by chemiluminescent detection with a CCD camera (LAS-3000, Fuji). Blots were subsequently gently stripped by 5 min incubation in 0.2 M NaOH at room temperature, followed by reprobing with monoclonal anti-phosphotyrosine antibody (2C8, Alexis Biochemicals) and finally with anti-Axl antibody to verify immunoprecipitation.

3. Results

3.1. RanBPM expression in human tissues and cancer cell lines

A portion of the cDNA sequence from the RanBPM yeast two-hybrid colony was utilised as probe for northern blot analyses of various human tissues and cancer cell line mRNAs (Fig. 1). RanBPM is widely expressed, as shown by detection of a single band at ∼3 kb in all human tissues tested. Highest transcript levels were detected in brain, heart, skeletal muscle, kidney and placenta, with low intestinal expression. Expression was also widespread amongst various cancer cells, being slightly higher in lymphoblastic leukaemia cells. Furthermore, expressed sequence tags (ESTs) for RanBPM have been isolated from many human tissues and tumour samples (NCBI EST database).

3.2. Isolation and identification of the Axl-binding protein in yeast as RanBPM

From our previous library scale yeast two-hybrid screen, we derived an Axl- (Fig. 2A) binding protein that was a fusion of yeast GAL4 activation domain

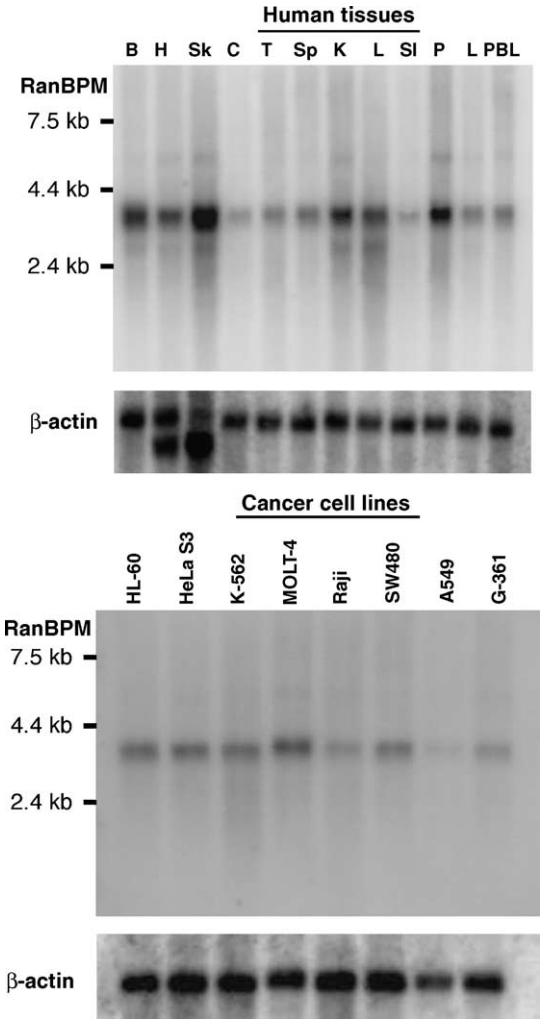


Fig. 1. Northern blot analyses of RanBPM mRNA in normal human tissues and cancer cell lines. A ^{32}P-labelled RanBPM cDNA probe was hybridised under high stringency to membranes containing equal amounts of mRNA from human tissues and human cancer cell lines. Lanes in human MTN northern blot are arranged as follows: B, brain; H, heart; Sk, skeletal muscle; C, colon; T, thymus; Sp, spleen; K, kidney; L, liver; SI, small intestine; P, placenta; L, lung; PBL, peripheral blood leukocytes. Lanes in human cancer cell line northern blot are arranged as follows: 1, promyelocytic leukaemia HL-60; 2, HeLa S3; 3, chronic myelogenous leukaemia K-562; 4, lymphoblastic leukaemia MOLT-4; 5, Burkitt's lymphoma (Raji); 6, colorectal adenocarcinoma SW480; 7, lung cancer A549; 8, melanoma G-361. The same membranes were stripped and reprobed for β-actin to verify equal RNA amounts (lower panels). Blots shown are representative of three independent hybridisations using separate membranes.

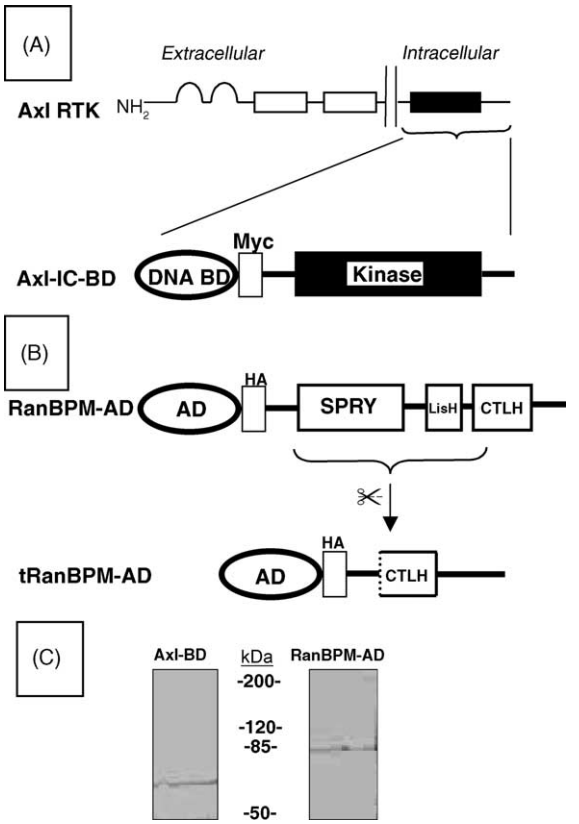


Fig. 2. Detection of RanBPM and Axl constructs in a positive yeast two-hybrid colony. (A) Schematic diagram of complete Axl RTK protein. For use in yeast two-hybrid studies, Axl intracellular domain (Axl-IC) was prepared fused to GAL4 DNA-binding domain (BD) and a c-Myc epitope tag. (B) Schematic diagram of RanBPM and truncated RanBPM (tRanBPM) constructs for yeast two-hybrid studies, prepared as fusions with N-terminal GAL4 activation domains (AD) and HA epitope tags. In tRanBPM, the SPRY and LisH motifs are absent. (C) Western blot detection of Axl-IC-BD and RanBPM-AD coexpressed in a yeast two-hybrid colony using antibodies against Myc and HA epitope tags, respectively.

(AD) and amino acids 136–729 of full length RanBPM (Genbank accession no. BAB62525; Fig. 2B), i.e. the remainder of the protein after an initial stretch of N-terminal glutamine and proline repeats. Here, we determined that this protein and the Axl-IC fused to Gal4 DNA-binding domain (DNA-BD) were present together in the same yeast colony by western blot detection, the proteins showing apparent molecular weights of 84 and 68 kDa, respectively (Fig. 2C).

3.3. Confirmation of in vivo association of RanBPM with Axl and Sky RTKs

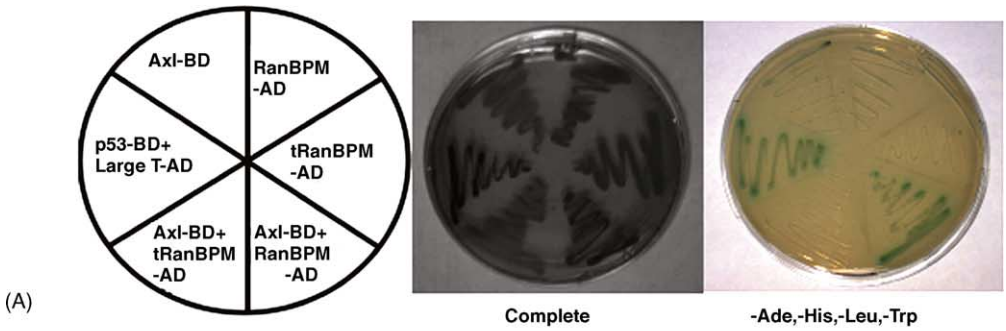
Further experiments in yeast confirmed the authenticity of the Axl–RanBPM interaction (Fig. 3A). Survival ability of yeast cells containing different constructs were compared, including yeast cells housing bait or partner constructs only. While all yeast constructs were able to grow on fully enriched YPDA medium, only coexpression of Axl-BD and RanBPM-AD conferred survival on quadruple dropout (QDO; -Ade, -His, -Leu, -Trp) agar plates, due to expression of all reporter genes. In contrast, a truncated form of RanBPM that omitted the entire SPRY and LisH domains did not confer survival, indicating that the Axl interaction occurs through this domain. In addition, the intracellular domain of Sky/Tyro3 RTK (Sky-IC-BD) was also able to interact with RanBPM-AD as shown by survival on QDO and concomitant activation of the α-galactosidase reporter gene (Fig. 3C).

3.4. Strength of interaction between Axl-IC and yeast two-hybrid binding partners

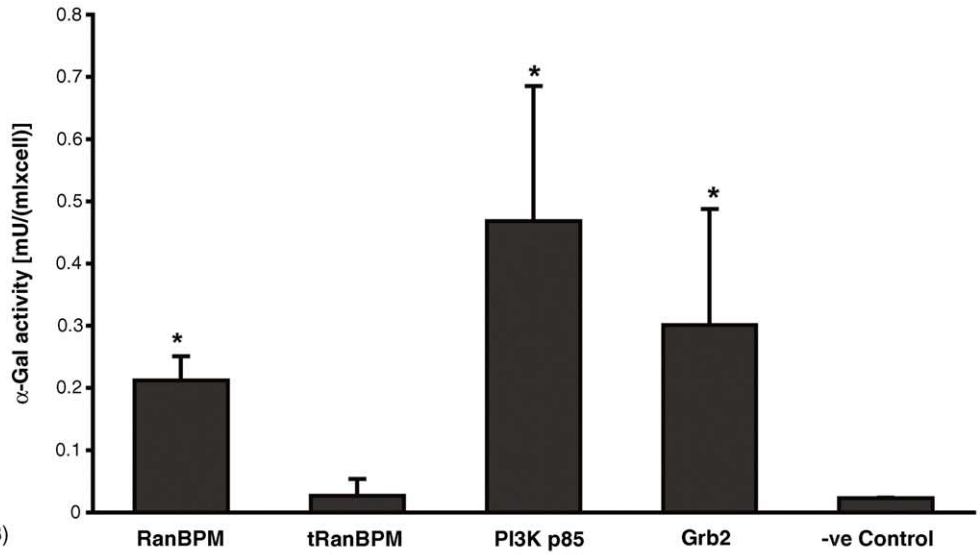
In order to determine the relative strength of yeast two-hybrid interactions, expression of the *MEL1*

Fig. 3. Qualitative and quantitative confirmation of yeast two-hybrid interaction between Axl, Sky and RanBPM. (A) Growth of yeast cells containing different protein constructs was compared by streaking onto complete nutrient (YPDA) and quadruple dropout (QDO; -Ade, -His, -Leu, -Trp) plates containing X-α-Gal. Whereas, all constructs grew on normal medium (middle panel), only Axl–RanBPM and positive control constructs thrived on QDO medium (right panel), showing expression of all reporter genes and α-Gal activity (blue colour). (B) Yeast cells containing various two-hybrid constructs were grown overnight in selective medium, after which cell-free supernatants were assayed for α-Gal activity as described. α-Gal activity was determined for the following yeast two-hybrid interactions: Axl–RanBPM, Axl–tRanBPM, Axl–PI3K (p85 subunit), Axl–Grb2, and lamin C-T antigen (negative control). Results shown are mean ± S.D. of α-Gal activity of three separate cultures for each construct assayed in triplicate. $^{*}P < 0.05$ vs. negative control. (C) The intracellular domain of Sky/Tyro3 RTK (Sky-IC-BD) also interacts with RanBPM-AD as shown by survival on QDO and concomitant activation of α-Gal (C). Three separate colonies were streaked per construct. Single construct controls on their own cannot confer survival on QDO. Blue spots in these controls are background colour production due to thickness of colonies picked at the start of the streak and are not true colonies. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

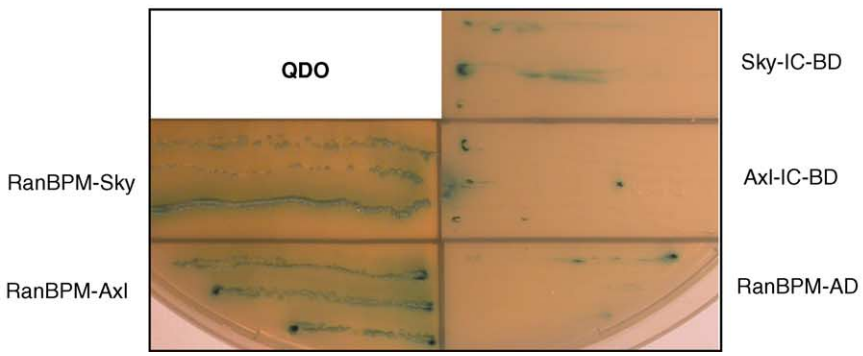
reporter gene was analysed through measurement of α-galactosidase (α-Gal) activity in yeast cell supernatants (Fig. 3B). The α-Gal activity of the Axl–RanBPM yeast culture was comparable to those of cultures containing the other identified Axl-binding signalling molecules PI3K and Grb2 (Braunger et al., 1997; Hafizi et al., 2002). In contrast, no activity was detected from yeast cultures coexpressing Axl and tRanBPM, as was the case with coexpression of lamin C-BD and large T antigen-AD (negative control).



(A)



(B)



(C)

3.5. Coimmunoprecipitation of RanBPM with Axl and vice versa

The interaction of RanBPM with Axl was reproduced in experiments where in vitro translated Axl-IC and RanBPM were coimmunoprecipitated using specific monoclonal antibodies against their epitope tags. In these experiments, proteins were synthesised in vitro lacking their respective GAL4 fusion domains but keeping their epitope tags for detection (Fig. 4A). Thus, RanBPM was detected in Axl immunoprecipitates and vice versa while the antibodies did not pull down the other tagged proteins alone. The cell-free nature of these experiments demonstrated the Axl–RanBPM interaction to be independent of phosphorylation.

Furthermore ectopically expressed RanBPM coimmunoprecipitated with full length endogenous Axl in mammalian COS cells (Fig. 4B). The anti-Axl antibody was not able to immunoprecipitate RanBPM-Myc overexpressed in HEK 293 cells, a cell line that does not express endogenous Axl (data not shown).

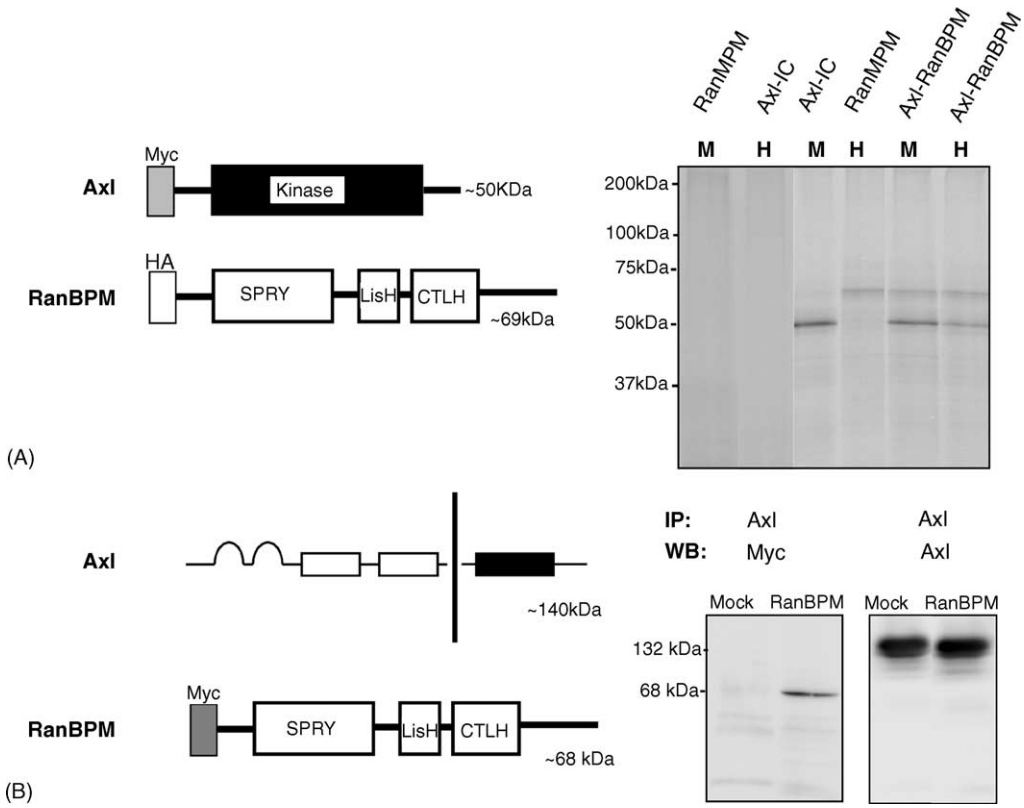


Fig. 4. Coimmunoprecipitation of Axl with RanBPM. (A) Interaction in a cell-free system. The coding cDNA sequences for Axl-IC and RanBPM that were utilised in the yeast two-hybrid system were translated in vitro to produce ^{35}S-labelled proteins lacking GAL4 domains but keeping their epitope tags. Translated products were incubated either alone or together, then subjected to immunoprecipitation using either monoclonal anti-Myc (M) or anti-HA (H) antibodies, followed by gel analysis as described. Presence of both Axl and RanBPM is observed from specific immunoprecipitation of each protein. As controls, anti-Myc antibody did not pull down HA-tagged protein alone and vice versa (lanes 1 and 2). Results shown are representative of three experiments with similar results. (B) Interaction in eukaryotic cells. COS cells, which endogenously express Axl, were transiently transfected with empty plasmid (mock) or plasmid encoding RanBPM containing an N-terminal Myc tag. Axl was immunoprecipitated from cell lysates using polyclonal anti-Axl antibody followed by western blotting using monoclonal anti-Myc antibody (lanes 1 and 2) or polyclonal anti-Axl antibody (lanes 3 and 4). Results shown are representative of three experiments with similar results.

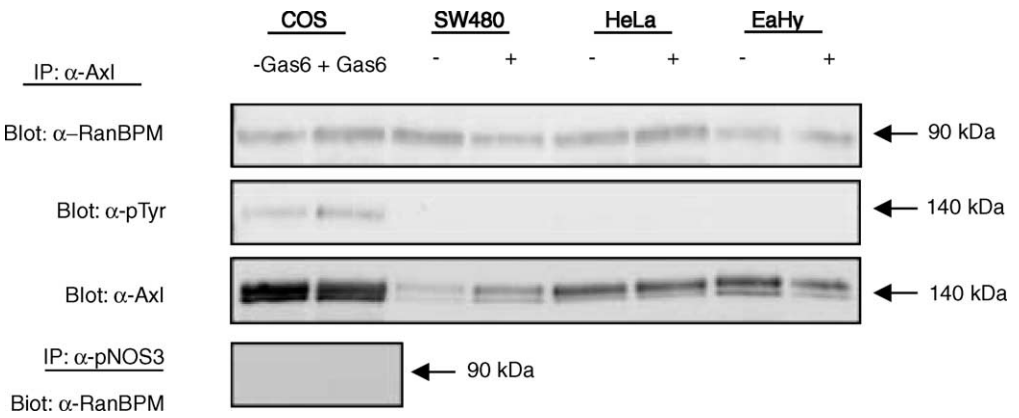


Fig. 5. Endogenous RanBPM interacts with endogenous Axl in various mammalian cell lines. Mammalian cell lines COS, SW480, HeLa S3 and EaHy were serum starved for 24 h then incubated for 45 min without and with recombinant human Gas6 (500 ng/ml). Cell lysates were subjected to overnight immunoprecipitation (IP) with polyclonal anti-Axl antibody. Immunoprecipitated proteins were subjected to 10% SDS-PAGE and western blotting using specific antibodies for detection of RanBPM (upper panel). Membranes were then, in turn, stripped and reprobed with antibodies against phosphotyrosine (middle panel) and Axl (lower panel). Immunoprecipitation with another goat polyclonal antibody yielded no RanBPM (negative control). Results shown are representative of two experiments with similar results.

3.6. Interaction of endogenous RanBPM and Axl proteins in mammalian cells

We also investigated the interaction of endogenous RanBPM with endogenous Axl in four mammalian cell lines that we show to express Axl: COS, SW480, Hela S3 and EaHy. Immunoprecipitation of Axl from precleared lysates from all four-cell lines revealed coimmunoprecipitation of full length 90 kDa endogenous RanBPM (Fig. 5). Immunoprecipitation with an unrelated control antibody of the same type and source as anti-Axl (anti-pNOS3) yielded no RanBPM. Stimulation of cells with recombinant human Gas6 caused a slight increase in tyrosine phosphorylation of Axl only in COS cells by roughly a third. We also observed tyrosine phosphorylation of Axl in response to Gas6 by first immunoprecipitating tyrosine phosphorylated proteins from the COS cell lysate and then detecting with anti-Axl antibody (not shown). The other cell lines did not respond to Gas6 stimulation, neither did they show altered association of Axl with RanBPM. We have previously demonstrated functionality of our recombinant human Gas6 protein, which we showed to both stimulate Axl activation and proliferation of mouse fibroblasts (Stenhoff, Dahlback, & Hafizi, 2004). Therefore, both endogenous RanBPM and Axl interact with each other in a constitutive manner. However, the interaction could not be correlated to Gas6 activation of Axl since the interaction occurred also to varying degrees in those cell lines showing no response to Gas6.

4. Discussion

Proteomic technologies are increasingly identifying novel intracellular proteins that physically interact with proteins of interest such as RTKs. For example, we first identified C1-TEN as a novel Axl-binding protein (Hafizi et al., 2002) and recently uncovered a negative regulatory function for it in terms of growth and migration signalling (Hafizi, Ibraimi, & Dahlback, 2005). In the present study, we have further characterised the novel intracellular interaction between Axl and RanBPM, therefore indicating the latter to play a role in regulation of Gas6–Axl signalling. After its original detection in the centrosome (Nakamura et al., 1998a), several novel RanBPM-protein interactions were subsequently reported, mainly with nuclear proteins (Bai et al., 2003; Greenbaum, Katcoff, Dou, Gozlan, & Malik, 2003; Ideguchi et al., 2002; Mikolajczyk, Shi, Vaillancourt, Sachs, & Nelson, 2003; Umeda, Nishitani, & Nishimoto, 2003; Wang et al., 2002b). However, more recent evidence has accumulated pointing to an additional role for RanBPM outside the nucleus, such as its interaction with MET RTK and subsequent regulation of the Ras/ERK signalling

pathway (Wang et al., 2002a). Their study was the first study to show an association between RanBPM and a protein at the level of the plasma membrane, thus precluding an exclusively nuclear localisation and role for RanBPM. However, it seems most evident that RanBPM is present in all cellular compartments, as reinforced in a recent study showing both cytoplasmic and nuclear localisation of RanBPM, where it interacts with the transcription factor p73 (Kramer et al., 2005). Here, we have also detected a cytoplasmic RanBPM interaction with the cytoplasmic portions of both related Axl and Sky RTKs.

We confirmed the authenticity of the Axl–RanBPM interaction and showed it to be comparable in strength to the Axl interaction with other well-established signal transduction molecules such as PI3K and Grb2. The Axl–RanBPM interaction was furthermore strong enough to be detected in vitro by coimmunoprecipitation experiments. As with all previous studies detecting RanBPM from expression libraries, we identified a shorter a version of RanBPM to bind to Axl in vivo and in vitro, which was the form that was identified to interact with Axl in the yeast two-hybrid studies. This construct houses the entire SPRY and LisH-CTLH domains, the SPRY and LisH domains appearing to be necessary for the interaction with Axl. Full length 90 kDa RanBPM contains an extra stretch of proline and glutamine residues at the N terminus, and is encoded by an extra 5′ region containing a large number of cytidine and guanidine nucleotides like many CpG islands throughout the genome and whose function is unclear. RanBPM has been detected in a large multiprotein complex of more than 670 kDa that also includes Ran GTPase (Nishitani et al., 2001) and Mirk (Zou, Lim, Lee, Deng, & Friedman, 2003). In order to verify the Axl–RanBPM as physiologically relevant, we investigated the interaction between the endogenous proteins in four mammalian cell lines that express Axl, the monomer protein being about 140 kDa in molecular weight. In all four-cell lines, full length RanBPM protein was demonstrated to interact with Axl protein.

Stimulation of cells with recombinant human Gas6 induced tyrosine phosphorylation of Axl only in COS cells. However, the RanBPM–Axl interaction could not be correlated to Gas6 activation of Axl since the interaction occurred to varying degrees also in those cell lines showing no response to Gas6. We have previously demonstrated that our recombinant human Gas6 protein is conformationally correct and functional, and stimulated Axl and ERK activation and proliferation in mouse fibroblasts (Stenhoff et al., 2004). Possible explanations for why only COS cells responded to exogenous Gas6 in terms of phosphorylation may be due to differences between cell lines in the levels of endogenous Axl expression, autocrine Gas6 stimulation, differences in regulatory signals further downstream, and sensitivity of our method. Nevertheless, it appears that both endogenous RanBPM and Axl interact with each other in a constitutive manner, as has been observed in the case of the interaction between RanBPM and MET RTK (Wang et al., 2002a). Thus, Gas6 stimulation of Axl per se may not be the principal input for alteration in the constitutive RanBPM–Axl interaction. It is also interesting to note observations on c-Mer, a sister receptor to Axl that interacted with the protein Vav1 constitutively and independently of phosphotyrosine, Vav1 actually being released from the receptor upon activation (Mahajan & Earp, 2003). Furthermore, we have also observed interaction between RanBPM and Sky, another member of the Axl RTK subfamily. Thus, a topic of further study would be to identify the exact region within all RTKs, potentially common and conserved, that interact with RanBPM.

The omission of the SPRY domain in RanBPM abolished binding to Axl altogether. An identical deletion strategy yielded the same result for the association of RanBPM with MET (Wang et al., 2002a). Within the region deleted was also a LisH motif shortly after the SPRY domain. This in RanBPM is a novel ubiquitous 34 amino acid motif that is found in 114 eukaryotic proteins, and which is apparently involved in homodimerisation (Kim et al., 2004). However, the SPRY domain is the most likely direct interaction site with Axl as RanBP10, a novel RanBPM-like protein with high amino acid sequence identity with RanBPM and that also interacts with MET RTK, does not possess a following LisH motif (Wang, Li, Schoen, Messing, & Wu, 2004). The SPRY domain has no known function currently identified. The high degree of amino acid sequence identity between all RTKs in their cytoplasmic regions (Manning et al., 2002) supports the recognition of a conserved region by the SPRY domain. The exact site of interaction within the RTKs is not known, although we found that phosphorylation of Axl was not essential for the interaction with RanBPM.

Expression of Axl has been shown to be prominent in various human tumours, including breast cancer (Berclaz et al., 2001), myeloid proliferative disorders (Neubauer et al., 1997), prostatic carcinoma (Jacob et al., 1999) and thyroid carcinoma (Ito et al., 2002). Furthermore, Axl and its biological ligand Gas6 have been implicated in cellular processes relevant to tumourigenesis such as survival, proliferation (Goruppi et al., 1996), migration and angiogenesis (Fridell et al., 1998). Therefore, knowledge on Gas6–Axl signalling may elucidate further the molecular mechanisms behind its role in tumourigenesis. Involvement of components of the PI3K pathway has been demonstrated, through a direct intracellular interaction of the PI3K p85 subunit with Axl (Braunger et al., 1997; Hafizi et al., 2002), and activation of both PI3K and its substrate Akt (Lee et al., 2002). Of particular significance is the observation that RanBPM activates the Ras/Erk pathway in MET RTK signalling (Wang et al., 2002a). This, together with the previous demonstration of a link between the Axl kinase domain and the Ras/Erk pathway (Fridell et al., 1996), therefore suggests that RanBPM may be a mediator between the Axl RTK and this particular signalling cascade.

The human RanBPM/RanBP9 gene is putatively composed of 14 exons spanning 90 kb on chromosome 6. RanBPM is highly conserved in both plant and animal kingdoms, with orthologues having been detected in many different species, and the mouse, hamster, and human forms of this protein being highly similar. Northern blot analyses revealed the expression of RanBPM to be widespread amongst normal human tissues, as well as in several different human cancer cell lines, although to varying extents. This near-ubiquitous expression profile is also apparent for Axl expression (O'Bryan et al., 1991), indicating that both proteins are important for normal cellular function. One study so far has provided evidence for a correlation between RanBPM and tumour development, where RanBPM expression was detected to be higher in tumour than in normal breast epithelial cell lines, as well as being frequently expressed in invasive breast carcinomas (Emberley et al., 2002). Although more work is required to probe this issue, it would nevertheless be of interest to determine whether RanBPM expression is correlated with expression or activity of Axl in breast cancer. In addition, expression of RanBPM in the brain also has relevance for its potential regulation of Sky RTK, which is expressed predominantly in the brain (Ohashi, Mizuno, Kuma, Miyata, & Nakamura, 1994). Furthermore, Sky RTK has been implicated in functions such as osteoclast bone resorbing activity (Nakamura et al., 1998b), Schwann cell proliferation (Li et al., 1996) and as a neurotrophic factor for hippocampal neurons (Funakoshi, Yonemasu, Nakano, Matumoto, & Nakamura, 2002). Therefore, the potential also exists for RanBPM involvement in normal and altered regulation of such processes.

In conclusion, we have identified a novel intracellular interaction between Axl RTK and RanBPM, occurring through the latter's SPRY and LisH domain region. These findings suggest roles for RanBPM in the cytoplasm beyond those that have tied it to the functions of Ran GTPase in the nucleus. Recently, RanBPM was shown to interact in the cytoplasm with fragile X mental retardation protein via a newly identified CRA motif at the C terminus of RanBPM (Menon, Gibson, & Pastore, 2004). Thus, RanBPM has emerged as a multimodular protein with the ability to interact with a host of proteins at different subcellular compartments and thereby regulate different roles. There is also an increasing body of knowledge indicating a peripheral membrane localisation for RanBPM, where it serves a scaffolding function, bridging interactions between the cytoplasmic domains of a variety of membrane proximal proteins. These include RTKs such as Axl and Sky (in the present study), MET (Wang et al., 2002a), the neurotrophin receptor (Bai et al., 2003) and β_2 integrin (Denti et al., 2004). The scaffolding function of RanBPM is further supported by its identification in a large multiprotein complex of more than 670 kDa (Nishitani et al., 2001). Therefore, stimulation of a protein that interacts with RanBPM, such as Axl or Sky RTKs, may allow closer interaction with other proteins in the complex via RanBPM. Further investigation of the influence of RanBPM on downstream components of the Gas6–Axl signal transduction pathway and their functional consequences is currently underway.

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