RanBPM Is a Phosphoprotein That Associates with the Plasma Membrane and Interacts with the Integrin LFA-1*

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Integrin adhesion receptors can act as signaling receptors that transmit information from the extracellular environment to the interior of the cell, affecting many fundamental cellular processes, such as cell motility, proliferation, differentiation, and survival. Integrin signaling depends on the formation of organized sub-membrane complexes that comprise cytoskeletal, adapter, and signaling molecules. The identification of molecules that interact with the cytoplasmic domain of integrins has been the focus of research aimed to elucidating the mechanistic basis of integrin signal transduction. We have identified RanBPM as a novel interactor of the β2 integrin LFA-1 in a yeast-two-hybrid screen. In the same assay, RanBPM also interacted with the β1 integrin cytoplasmic domain. We demonstrate that RanBPM is a peripheral membrane protein and that integrins and RanBPM interact in vitro and in vivo and co-localize at the cell membrane. We find that RanBPM is phosphorylated on serine residues; phosphorylation of RanBPM is increased by stress stimuli and decreased by treatment with the p38 kinase inhibitor SB203580. Transfection of RanBPM synergizes with LFA-1-mediated adhesion in the transcriptional activation of an AP-1-dependent promoter, indicating that the two proteins interact functionally as well. We suggest that RanBPM may constitute a molecular scaffold that contributes to coupling LFA-1 and other integrins with intracellular signaling pathways.

Integrins constitute a large family of receptors that mediate adhesive interactions between cells or between a cell and the extracellular matrix. Early characterization of integrins focused on the physical link they provide between the extracellular matrix and the cytoskeleton (1, 2). However, it has become apparent that integrin adhesion receptors regulate many other fundamental biological processes as well. In particular, integrins can act as signaling receptors that transmit information about the extracellular environment to the interior of the cell, affecting many important aspects of cell behavior, such as survival, proliferation, motility, and differentiation (3–7). Increasing evidence shows that integrin engagement can regulate transcription either through direct signaling to the nucleus (8, 9) or through biochemical elements shared with other signaling receptors, such as the Janus tyrosine kinase-signal transducers and activators of transcription or the MAPK1 pathways (9–13). Each integrin consists of a heterodimer of two transmembrane glycoproteins, an α and a β chain (14). Integrin LFA-1 (lymphocyte function-associated antigen-1), a heterodimer of the αL and the β2 subunits, is essential in many stages of the immune response, such as transmigration processes or the adhesive and signaling events that occur in the immunological synapse (7, 15, 16).

Integrin cytoplasmic domains are crucial in the establishment of organized complexes with the cytoskeleton (17), which contain many structural and signaling proteins and play a pivotal role in integrin signaling functions (18). Although the signal mediators activated by integrin engagement have been extensively characterized, the molecular mechanisms that link integrin receptors to the intracellular pathways are still under active investigation. To understand the physical basis of integrin-mediated signal transduction, we and others have focused our attention on the identification of proteins that interact with the intracellular domains (8, 18–21). We have searched for proteins that interact with the cytoplasmic domain of the β2 chain with the yeast two-hybrid system. Here we report the identification of RanBPM/RanBP9 as a β2-interacting protein. RanBPM was originally cloned for its ability to bind the small GTPase Ran and was reported to localize to the centrosome, a microtubule organization center. The centrosomal localization of RanBPM has not been confirmed by a subsequent report, which describes a cytoplasmic and nuclear distribution for the protein in the human KB carcinoma cell line. Ectopically expressed RanBPM was recently shown to interact with different signaling proteins, including the Met receptor, and to cooperate in regulating intracellular signaling pathways (22–24). In particular, overexpression of RanBPM was shown to induce activation of the Ras-Erk pathway and to activate transcription from the serum response element (22). However, both the cellular localization of the endogenous protein as well as the function of RanBPM are still unclear, because additional roles...
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**RanBPM Monoclonal Production**—To generate antibodies against RanBPM, amino acids 146–729 were cloned into pGEX-4T1 (EcoRI). The recombinant protein was purified on glutathione-Sepharose, dialyzed against phosphate-buffered saline (pH 7.4), and used to immunize mice. Anti-RanBPM antibodies were selected by their ability to recognize the antigen in enzyme-linked immunosorbent assays, and transfected Ran-BPM in Western blots and in immunofluorescence assays. 

**Cell and Tissue Lysates and Western Blot Analysis**—To obtain total cell lysates, cells were lysed in 150 mM NaCl, 10 mM Tris, pH 7.4, 1% Nonidet P-40, plus Pefabloc-SC (Roche Applied Science) and spun in a microfuge. Protein contents of the supernatant were measured by colorimetric reactions (Bradford method, Bio-Rad), and equal amounts of protein were resolved by SDS-PAGE, transferred to a membrane (Hybond-P, Amersham Biosciences), and hybridized with the RanBPM mAb. In the experiments shown in Figs. 3 and 4, lysis was carried out in the presence of a mixture of phosphatase inhibitors (Sigma). Tissues from 33- to 35-day-old C57 female mice were homogenized in solubilization buffer (25% v/v of Tris-HCl, 0.5 s, pH 6.8, 2.5% w/v of SDS) at 100 °C and spun at 13,000 rpm for 20 min, and protein was quantified with a bicinchoninic acid assay (Pierce).

Jurkat or COS7 cells were stimulated with anisomycin (50 ng/ml, Calbiochem), sorbitol (0.5 μM, Sigma-Aldrich), PMA (100 nM, Sigma) for 30 min or exposed to UV light (160–250 μJ/cm²), p38 MAPK inhibition was obtained by treating cells with SB203580 (25 μM, Calbiochem) for 45 min, prior to UV exposure. COST cells (60-mm diameter dish) were transfected with either wild-type or phosphorylation-deficient JNK1 or JNK1(α) (kind gift by R. J. Davis (27)). Samples were harvested after 48 h transfection.

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**Experimental Procedures**

**Two-hybrid Screen and Cloning of Ran-BPM and Vector Construction**—The entire cytoplasmic domain of the β₂ integrin, and of the αc chains of LFA-1 were fused to the GAL4-DNA binding domain (DBD) in the pAS1 vector (26). The constructs were tested for activation of a GAL4-dependent HIS3 and of a GAL4-dependent lacZ promoter in the Y153 and the YRG2 strains, in the presence of different concentrations of α-aminothiazole (3-AT), as described (26). The bait that did not exhibit any intrinsic activation function. Fusion protein expression was checked by Western blotting of yeast lysates with anti-GAL4-DBD antibodies. A human Epstein-Barr virus-transformed B cell library (kind gift from Steve Elledge), fused to the GAL4 activation domain in the yeast PACT vector was used for the two-hybrid screening in the presence of 25 mM 3-AT. Out of two million transformants screened, ten positive clones were obtained and plasmid DNA was isolated and used to transform the Y153 and YRG2 yeast strains in the presence of the β₂ cytoplasmic bait or of unrelated baits (αc, cyt, p53, large T antigen, DNA polymerase α) to test the specificity of the interaction.

Full-length RanBPM was obtained by screening a teratocarcinoma (NT-2) cDNA library in the Lambda-ZAP vector (Stratagene). Full-length RanBPM was subcloned into pcDNA-Amp for transient transfection in mammalian cells. FLAG-RanBPM was obtained by inserting an oligo encoding the FLAG epitope at the 3’ end of RanBPM cDNA. **In Vitro Protein-Protein Interaction Assays**—GST-β₂, GST-αc, and GST-p53 were obtained by subcloning the cytoplasmic domains of the αc and β₂ chains or p53 into pGEX-4T1 (Amersham Biosciences) (5). Proteins were expressed in Escherichia coli and purified according to the manufacturer’s protocol (Amersham Biosciences). In vitro transcribed and translated RanBPM was obtained with the TNT-treticucly lysate system (Promega), in the presence of [35S]methionine and cysteine. In vitro binding was performed as described (9). Briefly, 5 μl of labeled RanBPM was incubated with GST fusion proteins immobilized on glutathione-Sepharose beads for 1 h at 4 °C, then washed twice with 40 volumes of binding buffer (20 mM Hepes, pH 7.4, 400 mM NaCl, 0.25 mM MgCl₂, 0.5 mM diithiothreitol, 1% Nonidet P-40) and eluted with 25 mM glutathione. Eluted proteins were resolved on SDS-PAGE and visualized by autoradiography.

**Cell and Tissue Lysates and Western Blot Analysis**—To obtain total cell lysates, cells were lysed in 150 mM NaCl, 10 mM Tris, pH 7.4, 1% Nonidet P-40, plus Pefabloc-SC (Roche Applied Science) and spun in a microfuge. Protein contents of the supernatant were measured by colorimetric reactions (Bradford method, Bio-Rad), and equal amounts of protein were resolved by SDS-PAGE, transferred to a membrane (Hybond-P, Amersham Biosciences), and hybridized with the RanBPM mAb. In the experiments shown in Figs. 3 and 4, lysis was carried out in the presence of a mixture of phosphatase inhibitors (Sigma). Tissues from 33- to 35-day-old C57 female mice were homogenized in solubilization buffer (25% v/v of Tris-HCl, 0.5 s, pH 6.8, 2.5% w/v of SDS) at 100 °C and spun at 13,000 rpm for 20 min, and protein was quantified with a bicinchoninic acid assay (Pierce).

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**Metabolic Labeling and Pulse-chase Analysis**—COST cells were transfected in 60-mm dishes with the expression vector pcDNA1-RanBPM. After 48 h, cells were starved for 1 h in DMEM without methionine-cysteine, supplemented with 10% dialyzed PBS. Cells were pulsed with 200 μCi/ml [35S]methionine and cysteine (revidei Promix, Amersham Biosciences) for the indicated times. For chase experiments, COST cells were washed five times with PBS after pulsing and incubated with cold DMEM, 10% FBS for 2 or 3 h, as indicated.

Sorbitol (0.5 mM final) was added during the last 30 min of the pulse to the DMEM-medium of osteosarcoma Saos-2 cells to arrest cell cycle progression. Cell and Tissue Lysates were harvested in 150 μl of 50 mM Tris-0.5% SDS, boiled for 5 min at 95 °C, and adjusted to a final SDS concentration of 0.1% with RIPA correction buffer (25% v/v of Tris-HCl, 0.5M, pH 6.8, 2.5% w/v of SDS) at 100 °C and spun at 13,000 rpm for 20 min before immunoprecipitation.

**Phosphoamin Acid Analysis**—COST cells were transfected with pcDNA1-RanBPM. 48 h later, cells were incubated (2 h at 37 °C) with 1.25 mM CFP290-mm dish in 2 ml of phosphate-free medium. Osmotic shocked samples were exposed to 0.5 μM sorbitol for the last 30 min of the labeling period. Excess label was washed with ice-cold FBS, and cells were lysed in 50 mM Tris-0.5% SDS and treated with RIPA correction buffer and Pansorbin as above. Supernatants were immunoprecipitated with anti-RanBPM mAb. Immunoprecipitates were resolved by SDS-PAGE, transferred to polyvinylidene difluoro (Amersham Biosciences), and exposed to a radiographic film overnight. Labeled Ran-BPM was eluted from the membrane by treatment in 6 M HCl at 100 °C. The pellet was dissolved in distilled water, separated by thin layer chromatography, as described, and revealed by autoradiography.

**Subcellular Fractionation**—To obtain cytoplasmic fractions, freshly isolated T cells were treated for 7 min on ice with digitonin (Sigma) containing buffer (0.005% digitonin in 90 mM KAc, 25 mM Hepes, pH 7.4, 0.2 mM MgCl₂, 1 mM CaCl₂, 1 mM EGTA, 0.2 mM PMSF, and protease inhibitors). The digitonin-insoluble fraction was separated by ultracentrifugation at 100,000×g for 60 min (4 °C). Peripheral membrane proteins were extracted by treating the membrane pellets with 0.1 M Na₂CO₃, pH 11, followed by centrifugation at 150,000×g for 60 min. After removal
T lymphocytes were fixed in suspension and centrifuged on slides in a ferrin receptor (5E9C11), anti-PKC co-transformation of p53-DBD and large T antigen-AD as positive control. Cells were grown on His-3-AT-selective minimal medium.

mM Hepes, pH 7.4, 10 mM NaF, 5 mM MgCl2,1mM EGTA, 1% Nonidet with the GAL4 DNA binding domain (DBD):

Left in cold methanol/chloroform, blocked with 10% normal horse serum, Pathology Department of the San Raffaele Hospital. Sections were fixed an immunofluorescence microscope (Zeiss, Jena, Germany).

ates). All incubations were performed in PBS, 0.2% gelatin. Samples were analyzed with an MRC-1024 confocal microscope (Bio-Rad) or with an immunofluorescence microscope (Zeiss, Jena, Germany).

Sections of frozen tumor and normal tissues were obtained from the Pathology Department of the San Raffaele Hospital. Sections were fixed in cold methanol/chloroform, blocked with 10% normal horse serum, and incubated with anti-RanBPM mAb (5 μg/ml), followed by biotin-anti-IgG2a (Southern Biotechnology Associates) and Streptavidin-Cyanin2. LFA-1 was detected by mAb TS1.18 (anti-β2, IgG1), followed by fluorescein-goat anti-IgG1 (Southern Biotechnology Associates). All incubations were performed in PBS, 0.2% gelatin. Samples were analyzed with an MRC-1024 confocal microscope (Bio-Rad) or with an immunofluorescence microscope (Zeiss, Jena, Germany).

Immunoprecipitation—Jurkat cells were lysed in 150 mM NaCl, 25 mM Hepes, pH 7.4, 10 mM NaF, 5 mM MgCl2, 1 mM EGTA, 1% Nonidet P-40 with Complete protease inhibitors mixture (Roche Applied Science) and a phosphatase inhibitors mixture (Sigma) for 30 min in ice. 500 μg of total lysate was precleared with protein G-Sepharose for 1 h at 4°C and then immunoprecipitated for 2 h with 2 μg of antibody. Protein G-Sepharose was then added, and the mixture was incubated for an additional 1 h at 4°C. The beads were washed three times with lysis buffer, followed by elution in Laemmli buffer, SDS-PAGE, and Western blotting. Immunoprecipitations were carried out using the following antibodies: anti-LFA-1 (TS1.18 and TS1.22), anti-integrin β2 (polyclonal β2-cyto) (29), anti-CD4 (OKT3), anti-CD8 (S3.5), anti-transferrin receptor (2E9C11), anti-PRC/J (IC-16 sc-209, Santa Cruz Biotechnology). For Western blot analysis the same antibodies were used, except for anti CD3 zeta (6B10.2 sc-1239, Santa Cruz Biotechnology), anti-transferrin receptor (Zymed Laboratories Inc., 13–8600), and anti-integrin αL (610826 BD Transduction Laboratories).

Reporter Gene Assays—COS7 cells in 35-mm plates were transfected in triplicate samples with an AP-1 luciferase reporter gene, a β-galactosidase reporter construct, RSV-β-Gal, and either ICAM-1 or LFA-1 (αL, plus β2) constructs with or without 1.5 μg of full-length RanBPM. After 48 h cells were detached by trypsinization and plated on either anti-ICAM-1 or anti-LFA1 antibodies immobilized on non-tissue-culture treated plastic. Cells were stimulated for 2 h at 37°C and lysed, and luciferase activity in lysates was analyzed following the manufacturer’s instructions (Roche Applied Science). β-Galactosidase activity was measured in the same samples (Roche Applied Science).

RESULTS

Isolation of RanBPM and Interaction with LFA-1—To identify novel molecular intermediates of LFA-1 signaling, we used a yeast two-hybrid screen to search for proteins that interact with the cytoplasmic domain of the β2 chain of LFA-1 (β2cyt). The cDNA encoding the entire cytoplasmic domain of the β2 chain (amino acids 724–769) was cloned in-frame with the GAL4 DNA binding domain (DBD) in the pA51 vector and used to screen a GAL4 activation domain cDNA library derived from an Epstein-Barr virus-transformed B cell line (26). We identified two independent, overlapping cDNAs that specifically potentiated activation of a GAL4-dependent reporter in the presence of the β2-cyt-DBD fusion. Independent co-transformation of β2-cyt and the candidate clones confirmed the interaction (Fig. 1A). The specificity was demonstrated by lack of interaction with other control fusions, including the cytoplasmic domain of αL chain of LFA-1 (αLcyt), SV40 large T antigen, TrkB, the p180 catalytic subunit and the p49 primase subunit of DNA polymerase α (Fig. 1A and not shown). However, a bait construct consisting of the cytoplasmic domain of a highly related integrin, the β1 chain, also interacted with the candidate clones in the same assay. Sequencing of the cDNA inserts of the isolated clones revealed that they encoded a partial sequence of RanBPM/RanBP5, corresponding to the carboxyl terminus of the protein.

This cDNA insert was used to screen a cDNA library derived from the NT-2 cell line and to isolate a 2829-bp cDNA clone, which comprised the entire open reading frame of RanBPM. Northern blot analysis showed that RanBPM transcripts were widely expressed, as reported previously (22) and were also present in total RNA derived from normal lymphocyte cultures (not shown).

The full-length RanBPM clone was used to test the interaction with LFA-1 in an in vitro binding assay. In vitro transcribed and translated, 35S-labeled RanBPM specifically bound to β2-cyt fused to glutathione S-transferase (GST), but not to GST alone, nor to other unrelated fusion proteins, such as the cytoplasmic domain of the αL chain fused to GST (GST-αLcyt) or p53 (GST-p53, Fig. 1B).

Characterization of the Endogenous RanBPM Protein: Constitutive and Signal-induced Phosphorylation—To date, most studies have been performed on ectopically expressed RanBPM. Transfected RanBPM has been reported to distribute to the nucleus and the cytoplasm (30), however, the distribution and the properties of the endogenous protein have not been investigated in detail. To characterize endogenous RanBPM...
and its interaction with the LFA-1 receptor in mammalian cells, we generated monoclonal antibodies (mAbs) against recombinant RanBPM purified from E. coli. The anti-RanBPM mAb precipitated in vitro transcribed and translated RanBPM (Fig. 2A) and recognized a FLAG-tagged version of RanBPM transfected in COS7 cells (Fig. 2B, left lane). In addition, the antibody recognized a band of similar molecular weight in the untransfected cells, compatible with the endogenous protein (Fig. 2B, right lane). Consistently with the high degree of sequence conservation between murine and human RanBPM, our mAb recognized also the endogenous murine protein and detected RanBPM in all human and murine cell culture extracts (Fig. 2C) and in all murine tissue lysates analyzed (Fig. 2D). In several of these experiments, in addition to a major band, one or two higher molecular weight bands were recognized by the anti-RanBPM mAb.

Prediction analysis of RanBPM sequence for protein motifs (Scansite Motif Scan, available at scansite.mit.edu/) revealed the presence of putative consensus sites for phosphorylation by serine/threonine kinases, including sites (Ser/Thr-Pro) for proline-directed kinases (31), suggesting that the different migration species observed could be caused by different phosphorylation states of RanBPM. This hypothesis was supported by the finding that treatment of cells with stimuli (such as PMA, osmotic shock, and UV) known to activate proline-directed kinases of the MAPK family induced an increase of the upper molecular weight bands, compatible with a shift toward hyperphosphorylated forms of the protein (Fig. 3A). UV treatment and osmotic shock, in particular, were efficient stimuli to induce this pattern modification, suggesting that a stress-kinase, such as JNK or p38 MAPK kinase may be upstream of RanBPM modification. Consistently, the shift toward the upper band was reduced by pre-treatment of cells with an inhibitor of the p38 kinase (SB203580, Fig. 3B), but not by addition of an inhibitor of Erk1 activity (PD98059, not shown). We tested the possible involvement of JNK1 in RanBPM post-translational modification by transfecting COS7 with either a wild-type JNK1 construct or with a dominant-negative version of the same kinase (JNK1α1). Overexpression of the dominant-negative kinase did not affect the UV-induced molecular weight shift (Fig. 3C), indicating that RanBPM modification is independent of JNK1 activity.

To confirm that the slower migrating band represents a modification of RanBPM, we performed a metabolic labeling and pulse-chase experiment. COS7 cells were transfected with RanBPM, labeled with [35S]methionine and cysteine and then chased with fresh medium for the indicated times (Fig. 3D). RanBPM was immunoprecipitated from the cell lysates and analyzed by SDS-PAGE and autoradiography. In the absence of any stimulation, a slower migrating band appeared after the 60-min pulse and increased in intensity after a 120-min chase (Fig. 3D, left panel). A further increase was seen in cells exposed to osmotic shock (Fig. 3D, right panel). Treatment of the immunoprecipitates with alkaline phosphatase resulted in the complete disappearance of the slower migrating bands both in the unstimulated and in the stimulated samples (Fig. 3D, right panel), confirming that the slower migration pattern was indeed due to phosphorylation of the protein. Phosphoamino acid analysis of in vivo [35S]-labeled RanBPM demonstrated that both constitutive and signal-induced phosphorylations occur on serine residues (Fig. 3E).
Characterization of the Endogenous RanBPM Protein: Association with the Cell Membrane—Different subcellular expression patterns have been reported for RanBPM, including a centrosomal, a cytoplasmic, and a nuclear localization (30, 32). Since the membrane receptor LFA-1 interacted with RanBPM both in yeast cells and in vitro, we asked whether the subcellular distribution of endogenous RanBPM was compatible with such an interaction in mammalian cells in vivo. To analyze the subcellular distribution of endogenous RanBPM, we obtained digitonin-insoluble and digitonin-soluble (cytoplasmic) extracts from T lymphocytes and analyzed them for their RanBPM content. RanBPM was present both in the soluble and in the digitonin-insoluble fraction, indicating the existence of at least two distinct cellular pools of the protein (Fig. 4A). Interestingly, the slowest migrating (hyperphosphorylated) form of the protein partitioned exclusively in the digitonin-insoluble fraction.

We tested the nature of the RanBPM-insoluble pool by separating cytoplasmic and membrane fractions from post-nuclear Jurkat T cell lysates by ultracentrifugation. Western blot analysis showed that RanBPM was present in the cytoplasmic supernatant, as previously observed, was undetectable in the nuclear fraction, and was enriched in the membrane pellet, together with LFA-1 (Fig. 4B, left panel). Alkaline treatment of the membrane pellets released RanBPM from the pellet, as...
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Fig. 5. RanBPM associates with the cell membrane in cultured cells and in tissues in vivo. T lymphocytes (A), COS7 (B), and MDCK cells (C) were stained by indirect immunofluorescence with anti-RanBPM mAb, followed by fluorescein isothiocyanate-anti-mouse polyclonal antibody. Panels D–L show staining with anti-RanBPM mAb of sections from normal and cancer tissues (revealed either by 3-amino-9-ethylcarbazole, red, or diamobenzidine, brown, panels F and J). In polarized epithelial cells RanBPM has a basolateral distribution. D, normal lung, bronchial epithelium; E, normal kidney tubules; F, normal mammary gland. Staining for RanBPM is altered or lost in tumor tissues: G, lung carcinoma; H, kidney carcinoma; I, breast carcinoma. Panel J shows strong staining of a normal bronchus (bottom) and weak staining of the bronchogenic carcinoma cells (top). RanBPM positivity can also be detected in vivo in tissue resident macrophages (panel K) and tissue infiltrating lymphocytes (panel L). Immunofluorescent confocal analysis of LFA-1 (M, green) and RanBPM (N, red) in T lymphocytes from peripheral blood demonstrates a partial co-localization of the two proteins in membrane-associated clusters (yellow, panel O, overlay of panels M and N).

occurs for peripheral membrane proteins. In contrast, the transmembrane protein LFA-1 remained associated with the insoluble fraction also after alkaline treatment (Fig. 4B, right panel).

RanBPM Localizes to the Cell Membrane in Vivo—To confirm the membrane localization of RanBPM, we visualized RanBPM distribution in cultured cells by indirect immunofluorescence. RanBPM was visible in the cytoplasm and concentrated along the cell membrane in normal T lymphocytes, in COS7 cells, and in MDCK cells (Fig. 5, A–C, respectively). The study of RanBPM expression in human tissues confirmed that the same localization of the protein is present in vivo. Immunohistochemical analysis of tissue sections derived from surgical samples of human tumors and their normal counterparts revealed a similar distribution of RanBPM as the one previously observed in cultured cells: the protein was present along the cell membrane of normal lung (Fig. 5D), kidney (Fig. 5E), and breast epithelial cells (Fig. 5F), where it concentrated at the basal-lateral aspects of the cell membrane. In non-polarized cells, such as tissue-resident macrophages (Fig. 5K) or lymphocytes (Fig. 5L), RanBPM was distributed along the entire membrane of the cell.

Strikingly, decreased expression and loss of polarized distribution of the protein was detected in the cancer cells of a large number of tumor samples (Fig. 5, G–I, show lung, kidney, and breast cancer specimens, respectively). Fig. 5J shows, at smaller magnification, strong staining of a normal bronchus (bottom) compared with weak staining of lung carcinoma cells (top). In particular, 8 out of 15 lung carcinomas analyzed were negative for RanBPM, 5 showed weak positivity (less than 30% of tumor cells positive), and only 2 showed clear, although unpolared, RanBPM staining (not shown). In several cases, expression of RanBPM decreased as cancer cells moved away from the surrounding extracellular structures toward the center of a tumor nodule (Fig. 5O). The lowest levels of expression were seen in largely anaplastic tumors, such as those shown in Fig. 5, H and I.

Endogenous RanBPM Interacts with β2 and β3 Integrins in Vivo—The membrane localization of RanBPM in lymphocytes encouraged us to further investigate the interaction between RanBPM and LFA-1. In an indirect immunofluorescence assay the two proteins showed partial co-localization in cell membrane clusters in normal human T lymphocytes (Fig. 5, M–O). Co-immunoprecipitation experiments were also performed in the Jurkat T cell line to investigate the interaction of endogenous RanBPM with integrins in vivo. Cell lysates were subjected to immunoprecipitation with anti-β2- and -β3 integrin antibodies, as well as with antibodies recognizing several additional transmembrane or cytosolic molecules. We found that anti-integrin antibodies co-precipitate endogenous RanBPM protein under rather stringent conditions (1% Nonidet P-40, Fig. 6A), and the interaction was maintained in the presence of an alkylating agent (N-ethylmaleimide, 10 mM) that prevents disulfide bond formation (not shown). In anti-LFA1 immunoprecipitates both the phosphorylated and the unphosphorylated form of RanBPM were consistently present. Low levels of RanBPM were also detected in immunoprecipitates of several additional transmembrane receptors tested, including CD4, CD71 (transferrin receptor), and MHC class I molecules (Fig. 6A and data not shown). Conversely, selected transmembrane (CD3ε) or cytosolic (PKCβ) molecules failed to associate with RanBPM in co-immunoprecipitation experiments. The same membranes were reprobed with antibodies against the various immunoprecipitated proteins. Eluates did not contain contaminating LFA-1 proteins, with the exception of the anti-CD71 immunoprecipitates, which reproducibly contained β2 integrins (Fig. 6A). A direct association between integrin chains and the transferrin receptor has been previously reported for the integrin α3 (33).

Expression of RanBPM Potentiates LFA-1-dependent Activation of an AP-1 Promoter—The function of RanBPM is still under investigation, however, a role for RanBPM in the control of transcriptional activity in different systems has been recently proposed (22, 25). In particular, overexpression of RanBPM in HeLa cells has been reported to induce the expression of a serum response element reporter gene. We and others (4, 8, 34–36) have shown that integrin receptor signaling can regulate the transcriptional activity of the AP-1 complex. Thus, to test the functional consequences of the interaction between RanBPM and LFA-1 we asked whether expression of RanBPM could modulate AP-1-dependent transcription induced by LFA-1 signaling. We transfected COS7 cells with c-Jun, an AP-1 luciferase reporter gene, and LFA-1 or another lymphocyte cell surface molecule (ICAM-1), as a control. Cells were stimulated by plating on immobilized anti-LFA-1 or anti-ICAM-1 mAb, respectively. Under the conditions chosen for
Integrin receptors are involved in multiple signal transduction processes that critically regulate cell behavior and fate. Many of these events require changes in gene expression that are induced by integrins through a variety of mechanisms, which include activation of signaling mediators, nuclear translocation of proteins, or mRNA stabilization (10, 11, 37, 38). Integrins are not directly endowed with enzymatic properties and translate information to the intracellular signaling pathways by recruiting adapter and cytoskeletal proteins to their cytosolic domains. Leukocytes have the ability to establish integrin-mediated interactions with other cells and the extracellular matrix, which are essential for leukocyte differentiation and effector functions. In the search for molecular intermediates of integrin LFA-1 signaling we have identified RanBPM as an integrin-binding protein. The biochemical interaction between RanBPM and LFA-1 was confirmed by colocalization and co-immunoprecipitation studies in T lymphocytes and supported the functional interaction we detected between LFA-1 and RanBPM in the activation of the AP-1 transcriptional pathway. These findings indicate that RanBPM may be involved in coupling LFA-1 with intracellular signaling pathways. In addition, RanBPM may have a role in different integrin signaling pathways, as suggested by the interaction with the highly related β1 cytoplasmic domain.

Although originally cloned for its ability to bind the small GTPase Ran and proposed as a novel regulator of microtubule polymerization (32), RanBPM was subsequently reported to interact both in vitro and in vivo with a broad spectrum of functionally unrelated transmembrane and intracellular molecules. In particular, novel roles for RanBPM have been suggested by its ability to interact with receptors and to affect their signaling properties and transcriptional activities (22, 25). Among RanBPM-interacting receptors are the steroid hormone receptors (25), the tyrosine kinase receptor Met (22), the Igβ antigen receptor on B cells (T. Doi and T. Watanabe, GeneBank entry NP_064314), the p75 neurotrophin receptor (24), and the G protein-coupled V2 vasopressin receptor.² Because most of the above interactions have been detected in the yeast two-hybrid system and confirmed in vivo by overexpressing the molecules involved, we sought to analyze the interaction of integrins with endogenous RanBPM. Our results confirmed the existence of such interaction, although it does appear, based on our findings, that RanBPM is a rather promiscuous adaptor for several transmembrane receptors. These findings, together with RanBPM localization at the plasma membrane, are consistent with a possible role of RanBPM as a scaffolding protein. Interestingly, RanBPM was found in a large multiprotein complex in co-immunoprecipitation and gel filtration assays (30). The protein muskelin has been identified as part of the RanBPM complex (39). Muskelin has been cloned for its ability to alter cell adhesion and to affect cytoskeletal organization and focal contact dynamics (40). Muskelin subcellular localization (cytoplasmic, with localized concentrations at the cell membrane) is consistent with the localization of RanBPM described in our study. The co-segregation of these proteins in the same multi-molecular complex may reflect their role as molecular links between integrins and the cytoskeleton. The ability of RanBPM to interact with multiple membrane-associated proteins may be of great relevance for integrin-mediated signaling, because one of the most biologically significant aspects of integrin function is the ability to modulate signaling cascades initiated by other receptors, thereby integrating positional and structural cues with information provided by soluble factors (11, 36, 41–43). Many signaling interactions between integrins and receptor tyrosine kinases or G protein-coupled receptors (GPCRs) have been described, including receptor transactivation, synergy in

² G. Innamorati, unpublished data.
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the transmission of signals between the cytoplasm and the nucleus, or reciprocal modulation of downstream pathways (43). One example is the activation of the Erk/MAPK pathway by growth factors or GPCRs, which requires the simultaneous engagement of adhesion receptors (35, 36, 38). In addition, signaling from GPCRs, such as vasopressin or bradykinin receptors, has been shown to converge with integrin-generated signaling on the activation of focal adhesion kinase (44). Also in the case of the Met receptor, a bidirectional cross-talk with adhesion receptors has been described, resulting both in the modulation of Met signaling by integrin engagement (45) and the enhancement of integrin clustering and cytoskeletal interactions induced by Met activation (46, 47). Although in most cases the mechanistic basis of receptor cross-talk is unclear, there is growing awareness of the role played by receptor and signal mediator compartmentalization, which takes place through the assembly of spatially organized scaffolds. The ability of RanBPM to interact both with adhesion and “classic” signaling receptors, such as Met, together with its association with the plasma membrane, places RanBPM in the ideal position to act as a molecular platform that brings together different signaling pathways.

Many protein interactions are regulated by post-translational modifications, and it is intriguing to speculate that the phosphorylation state of RanBPM may play a significant role in determining the localization of endogenous RanBPM, as suggested by the partitioning of phosphorylated RanBPM to the digitonin-insoluble fraction; however, the causal relationship between serine phosphorylation of RanBPM and its localization on the plasma membrane remains to be established. Additional roles for RanBPM phosphorylation can be also envisioned, such as the regulation of interactions with other signaling elements in response to extracellular stimuli like UV and osmotic shock.

The coordination of signals generated from adhesion receptors with other environmental cues is essential for the functional and structural integrity of a tissue: perturbations of this cross-talk are characteristic of aberrant cell behavior such as seen in malignant transformation. The alteration of the RanBPM expression pattern observed in tumor samples may reflect the disrupted coordination between adhesive processes and intracellular signaling, which typically occurs in cancer cells.

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