Tumor Cell Pseudopodial Protrusions

LOCALIZED SIGNALING DOMAINS COORDINATING CYTOSKELETON REMODELING, CELL ADHESION, GLYCOLYSIS, RNA TRANSLOCATION, AND PROTEIN TRANSLATION*

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The pseudopodial protrusions of Moloney sarcoma virus (MSV)-Madin-Darby canine kidney (MDCK)-invasive (INV) variant cells were purified on 1-µm pore polycarbonate filters that selectively allow passage of the pseudopodial domains but not the cell body. The purified pseudopodial fraction contains phosphorysorinated proteins, including Met and FAK, and various signaling proteins, including Raf1, MEK1, ERK2, PKBα (Akt1), GSK3α, GSK3β, Rb, and Stat3. Pseudopodial proteins identified by liquid chromatography tandem mass spectrometry included actin and actin-regulatory proteins (ERM, calpain, vinculin, talin, and β1 integrin), glycolytic enzymes, proteins associated with protein translation, RNA translocation, and ubiquitin-mediated protein degradation, as well as protein chaperones (HSP90 and HSC70) and signaling proteins (RhoGDI and ROCK). Inhibitors of MEK1 (U0126) and HSP90 (geldanamycin) significantly reduced MSV-MDCK-INV cell motility and pseudopod expression, and geldanamycin treatment inhibited Met phosphorylation and induced the expression of actin stress fibers. ROCK inhibition did not inhibit cell motility but transformed the pseudopodial protrusions of MSV-MDCK-INV cells into extended lamellipodia. Dominant negative Rho disrupted pseudopod expression and, in serum-starved cells, 1-α-lysophosphatidic acid (oleoyl) activation of Rho induced pseudopodial protrusions or, in the presence of the ROCK inhibitor, extended lamellipodia. RNA was localized to the actin-rich pseudopodial domains of MSV-MDCK-INV cells, but the extent of colocalization with dense actin ruffles was reduced in the extended lamellipodia formed upon ROCK inhibition. Rho/ROCK activation in epithelial tumor cells therefore regulates RNA translocation to a pseudopodial domain that contains proteins involved in signaling, cytoskeleton remodeling, cell adhesion, glycolysis, and protein translation and degradation.

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The repeated, directional extension and stabilization by substrate adhesive contacts of pseudopodia constitute the basic mechanism by which cells move over a substrate (1). Cell motility is therefore associated with the polarized formation of a distinct plasma membrane domain, the pseudopod, whose stabilization determines the directionality of cell movement (2). Receptor stimulation of Rho GTPases and phosphatidylinositol 4,5-bisphosphate (PIP2) activates WASp/Scar proteins at the leading edge recruiting Arp2/3 and actin monomers to induce actin filament branching and membrane protrusion (3). Complex interactions between these signaling modules, and others, are implicated in the protrusive and adhesive modes of cell migration.

MSV-MDCK-INV cells, selected from Moloney sarcoma virus (MSV)-transformed MDCK cells for their ability to invade across Matrigel-coated filters, exhibit multiple protrusive, actin-rich, blebbed pseudopodial domains whose formation is dependent on autocrine Met activation (4–6). Using polycarbonate filters of defined 1-µm pore size that selectively allow passage of the pseudopodial domains but not the cell body, we established a method to purify MSV-MDCK-INV pseudopodia (6). The pseudopodia of MSV-MDCK-INV cells are strongly labeled for β-actin by immunofluorescence, and the purified pseudopodial fraction (PPF) is enriched for β-actin and depleted of mitochondrial proteins relative to the total cell lysate (4, 6). Glycolysis represents a critical energy supply for tumor cell motility (7), and identification of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase by Edman degradation in purified pseudopodia of MSV-MDCK-INV cells led to the demonstration that glycolysis regulates the formation and protrusion of tumor cell pseudopodia (6).

Using a scaled up pseudopod purification approach, we show here that the pseudopodial domain of MSV-MDCK-INV cells contains phosphorylated Met and FAK as well as a number of downstream signaling proteins, including Raf1, MEK1, ERK2, PKBα (Akt1), GSK3α, GSK3β, Rb, and Stat3. A subsequent proteomic analysis identified other regulators of Met activity, 1-

1 The abbreviations used are: MSV, Moloney sarcoma virus; HGF, hepatocyte growth factor; HGFR, hepatocyte growth factor receptor; p-, phospho-; p-Tyr, phosphotyrosine; MAPK, mitogen-activated protein kinase; ROCK, Rho kinase; p97(VCP), valosin-containing protein; EF1α, elongation factor 1α; NPC, nuclear pore complex proteins; ERM, ezrin/radixin/moesin; LPA, 1-α-lysophosphatidic acid, oleoyl; MDCK, Madin-Darby canine kidney cells; PPF, purified pseudopodial fraction; GFP, green fluorescent protein; EGFP, enhanced GFP; MOPS, 4-morpholinoethanesulfonic acid; MS, mass spectrometry; ER, endoplasmic reticulum; DN, dominant negative; ERK, extracellular signal-regulated kinase; INV, invasive.
including RhodGDI, ROCK2, and HSP90, as well as major classes of proteins that included cytoskeleton-associated proteins, such as actin, tubulin, vimentin, and actin- and tubulin-associated proteins, glycolytic enzymes and protein chaperones, as well as proteins associated with RNA translocation, protein translation, and ubiquitin-mediated protein degradation. Pseudopodial protrusion was inhibited by the MEK1 inhibitor U0126 and by inhibition of HSP90 activity with geldanamycin, which was also shown to inhibit constitutive Met activation. ROCK inhibition with Y27632 did not affect cell motility but transformed the protrusive pseudopodia into extended lamellipodia. Inhibition of ROCK was associated with reduced RNA targeting to the lamellipodia showing that Rho/ROCK activation recruits RNA to the pseudopodial domain of MSV-MDCK-INV cells for local translation.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents—**Anti-β-actin, anti-BiP/GRP78, and anti-talin were purchased from Sigma; anti-FAK and anti-phosphotyrosine (p-Tyr; PY-99), anti-phospho-RB1 (S780), anti-HSC70, and anti-lamin A/C were from Santa Cruz Biotechnology; anti-phospho-Met (Y1230, Y1234, and Y1255), anti-phospho-FAK Y397, and anti-phospho-FAK Y377 were from BIOSOURCE International; anti-p97(VCP) was from Abcam Inc.; anti-calpain 2 and anti-paxillin were from Calbiochem; anti-HSP90 was from Stressgen; anti-EGF1α was from Upstate; anti-nuclear pore complex proteins (NPC; Mab414) were from Covance Research Products; anti-vinculin, anti-α5β1 integrin, and anti-GAPDH were from Chemicon Inc.; and anti-mitochondrial HSP70 was from Affinity Bioreagents. pEGFP-actin plasmid was purchased from BD Biosciences, and Effectene transfection reagent was from Qiagen. 24-mm Falcon polycarbonate filters (1-µm pore size) were purchased from VWR, and 100-mm 0.4-µm pore filters were labeled with Sytox green to label the nuclei (A) of MSV-MDCK-INV cells. MSV-MDCK-INV cells were transfected with pEGFP-actin (B). Texas Red phalloidin was used for F-actin (B), and PY-99 followed by Alexa633-conjugated secondary antibody to identify phosphotyrosinated proteins (C). Z-scans show the top of the filter to the left and the bottom of the filter of the dashed line that corresponds to the filter. Merged images (D) show nuclei in green, actin in red, and p-Tyr in blue. E, equal protein equivalents (10 µg) of MSV-MDCK-INV cell lysates (INV), and purified pseudopodia fraction (PPF) were blotted for p-Tyr (left-hand blot) or for FAK and phospho-HGF/Met (p-Met) (right-hand blot). MSV-MDCK-INV cells were immunofluorescently labeled for p-Met Y1230, Y1234, Y1255 (F) and p-FAK Y397 (H) as well as for F-actin with phalloidin (G and I). A similar pattern of labeling was obtained with antibodies to p-FAK Y397 and p-FAK Y576. Bar, 10 µm.

**Immunofluorescence Labeling—**Immunofluorescence labeling of cells plated sparsely for 1 day on glass coverslips or on 1-µm pore filters (Falcon) was performed as previously described (6). Cells were fixed with 3% paraformaldehyde and permeabilized with 0.2% Triton X-100, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 0.05 mM p-nitrophenyl-p’-guanidino benzoxate, 10 µg/ml leupeptin, pepstatin A, and aprotinin). The recovered PPF was sonicated twice for 15 s on ice then centrifuged for 30 min at 150,000 rpm, and the supernatant was collected and the protein concentration was measured (BCA assay, Pierce). Samples used for the Kinetworks™ KPSS 1.3 Phosphosite Protein Screen (Kinexus Bioinformatics Corp., Vancouver, BC) were immediately boiled in Laemmli sample buffer and shipped at room temperature as per the company’s instructions (www.kinexus.ca).

**Phosphotyrosine-rich pseudopodia of MSV-MDCK-INV cells selectively pass through 1-µm pore filters.** MSV-MDCK-INV cells plated on 1-µm pore filters were labeled with Sytox green to label the nuclei (A), Texas Red phalloidin to label F-actin (B), and PY-99 followed by Alexa633-conjugated secondary antibody to identify phosphotyrosinated proteins (C). Z-scans show the top of the filter to the left and the bottom of the filter of the dashed line that corresponds to the filter. Merged images (D) show nuclei in green, actin in red, and p-Tyr in blue. E, equal protein equivalents (10 µg) of MSV-MDCK-INV cell lysates (INV), and purified pseudopodia fraction (PPF) were blotted for p-Tyr (left-hand blot) or for FAK and phospho-HGF/Met (p-Met) (right-hand blot). MSV-MDCK-INV cells were immunofluorescently labeled for p-Met Y1230, Y1234, Y1255 (F) and p-FAK Y397 (H) as well as for F-actin with phalloidin (G and I). A similar pattern of labeling was obtained with antibodies to p-FAK Y397 and p-FAK Y576. Bar, 10 µm.
cells from a minimum of two experiments were analyzed per protein. Transient GFP transfections were performed for 20–24 h to limit overexpression of the transfected protein.

**SDS-PAGE and Immunoblotting**—MSV-MDCK-INV cells cultured overnight to 50–60% confluency were scraped, lysed, and sonicated in ice-cold lysis buffer as described above. Protein content of cell lysates and the cell body and pseudopodial fractions (PPFs) of MSV-MDCK-INV cells was determined using the BCA protein assay (Fierce). 10–20 μg of protein was separated by SDS-PAGE and blotted onto nitrocellulose paper and blocked with 5% milk or, for phosphoprotein blots, with 4% bovine serum albumin, prior to probing with the appropriate primary antibodies and secondary antibodies conjugated to horseradish peroxidase. The labeled bands were revealed by chemiluminescence and exposed to Kodak XRP-1 film.

**Mass Spectrometry**—The PPF (190 μg) was separated on an 8% SDS-PAGE gel, and excised gel bands were digested by Trypsin (Promega, Madison, WI) or ProGest Digestion Robot (Genomic Solutions, Ann Arbor, MI) according to the protocol described in Ref. 8. After lyophilization, tryptic peptides were analyzed by liquid chromatography-mass spectrometry. Liquid chromatography-mass spectrometry consisted of Ultimate high-performance liquid chromatography system (Thermo Electron, San Jose, CA). Peptides were separated on custom-made 75-μm i.d. PicoTip columns packed with 3-μm C-18 beads. The column effluent was sprayed directly into the mass spectrometer. The gradient used for separation was 3–60% of acetonitrile during 25 min, and the total run time was 45 min. Raw mass spectrometric data were screened against the NCBI data base using the Mascot search engine (Matrixscience, London, UK). A protein was considered as a hit if two independent peptide matches to individual scores higher than 40 were found. Single peptides with a score higher than 40 were validated manually based on their tandem mass spectrometry fragmentation.

**RESULTS**

**Purification of Tumor Cell Pseudopodia**—MSV-MDCK-INV pseudopodia can be purified by plating the cells on polycarbonate filters of 1-μm pore diameter that permit the selective passage of MSV-MDCK-INV pseudopodia but not the cell body (6). As seen in Fig. 1 A–D), the nucleus and actin-labeled cell body remain on the upper surface of the filter while actin-labeled protrusions pass through to the underneath of the filter. The pseudopodial protrusions that pass through the filter are labeled for phosphotyrosine (p-Tyr). Our prior studies on pseudopodia purification used 24-mm filter units (6), and we have scaled up the purification procedure using 100-mm diameter filters (for details see “Materials and Methods”). MSV-MDCK-INV cells exhibit increased total phosphotyrosine expression that concentrates in the pseudopodial domain (5), and we assessed the pattern of phosphotyrosine phosphorylation in the pseudopodial fraction by blotting with the anti-p-Tyr antibody PY-99 (Fig. 1 E). Only slight differences in the phosphotyrosine expression pattern between the pseudopodial fraction and the cell lysate were observed. The major bands in both fractions migrated at 160, 125, and 60 kDa. Blotting with antibodies to phosphorylated HGF/Met (p-Met Y1230, Y1234, and Y1235) and to FAK revealed that they comigrate with major phosphorylated bands at 160 and 125 kDa, respectively (Fig. 1 E). By immunofluorescence, p-Met and p-FAK colocalize with actin and p-Tyr in the pseudopodial domain (Fig. 1 F–I). Activated forms of both Met and FAK are therefore present in MSV-MDCK-INV pseudopodia.

We subsequently analyzed the PPF for expression of the phosphorylated forms of various signaling proteins using a panel of 31 different phospho-site antibodies with the Kinetworks™ KPSS-1.3 Screen. As seen in Fig. 2 A, MSV-MDCK-INV pseudopodia express the phosphorylated forms of multiple signaling proteins consistent with localized autocrine Met activation, including Src, MEK1/2, ERK1/2, GSK3α/β, PKBα/β, Akt1, PKCo, Rb, and STAT3. Downstream Met signaling cascades are therefore active within the pseudopodia of MSV-MDCK-INV cells. The phospho-site screen also identified phosphorylated adducin-α, JNK, Rafl, and STAT1 as enriched in the pseudopodial fraction (Fig. 2 B).

**The Pseudopod Proteome**—A subsequent proteomic analysis of the pseudopodial fraction by liquid chromatography tandem mass spectrometry analysis identified 167 proteins from 34 Coomasie-identified bands (Table I). Bovine serum albumin and other serum proteins were picked up in the screen, and proteins of potential serum origin were deleted from the analysis. Identified proteins were classed into major families, including cytoskeleton-associated proteins (28 proteins), adhesion proteins (5 proteins), glycolytic enzymes (13 proteins), protein chaperones (10 proteins), translation-associated proteins (23 proteins), RNA-binding proteins (14), ubiquitin/pro-
teasome-associated proteins (11 proteins), signaling proteins (16 proteins), as well as proteins associated with membrane trafficking (6 proteins) and cellular organelles (13 proteins). Twenty-eight proteins could not be classified into these categories (Table I). Evidence for the selective nature of the domain that passes through the filter is evident from the abundance of actin, actin-associated proteins, tubulin, and glycolytic enzymes. The proteomic approach will necessarily pick up the most abundant proteins, and a crude indication of abundance can be obtained from the number of peptides identified that correspond to a specific protein. Although the molecular size of a protein will necessarily impact on the number of peptides identified, assessment of peptide number per class indicates clearly that the pseudopodial fraction is enriched for cytoskeletal proteins, glycolytic enzymes, protein chaperones, and translation associated proteins (Fig. 3). Based on the number of peptides the most abundant proteins in the pseudopodial fraction in addition to actin include the ERM proteins (ezrin, moesin, and radixin), myosin IX and VI, tubulin, vimentin, various glycolytic enzymes, the chaperones HSP90, HSC70, and CCT, elongation factors 1 and 2, the VCP/p97 AAA ATPase, and the signaling proteins ULIP and Sra1.

### Table 1

| Protein Type | Number of Proteins | Number of Peptides |
|--------------|--------------------|--------------------|
| Actin-associated | 11 | 15 |
| Signaling | 16 | 20 |
| Membrane trafficking | 6 | 10 |
| Cellular organelles | 13 | 18 |

The number of proteins per class is given in parentheses, and the number of peptides is given in adjacent columns.
Proteomic Analysis of Tumor Cell Pseudopodia

We confirmed the presence of a number of the major pseudopod-associated proteins by immunoblotting of the PPF and cell body fractions (Fig. 4). EF1α, HSP90, HSC70, calpain2, vinculin, paxillin, FAK, p-FAK, p97/VCP, glyceraldehyde-3-phosphate dehydrogenase, and NPC are present in the pseudopodial fraction at levels corresponding to that of β-actin with calpain and paxillin exhibiting enrichment in the pseudopodial domain relative to actin. Faint bands for BiP and lamin A/C were detected in the PPF, and quantification showed that pseudopodial expression of these two proteins was minimal relative to the cell body and equivalent to that of mitochondrial HSP70, previously shown to be excluded from MSV-MDCK-INV pseudopodia (6).

We subsequently used quantitative immunofluorescence to assess protein distribution to the actin-defined pseudopodial domain relative to the rest of the cell (Fig. 5). All the proteins exhibited a diffuse cytoplasmic labeling to varying extents, nevertheless distinct distributions of the proteins to the pseudopodial domain were detected. Pseudopodial expression was quantified by comparing average pixel density in the phalloidin-labeled pseudopodial domain relative to the rest of the cell. To ensure that protein distribution to pseudopodia is not a volume effect and due to diffusion of cytosolic proteins, we transfected cells with GFP that exhibited a reduced density in the actin-dense pseudopodial domain relative to the rest of the cell. As seen in Fig. 5Q, a number of proteins (vinculin, paxillin, phospho-FAK, calpain 2, talin, α5β1 integrin, EF1α, and pRb1) exhibited significant enrichment in the pseudopodia relative to GFP, whereas others exhibited a similar ratio (FAK, HSP70, HSC70, p97, and NPC) and others were depleted (lamin A/C, BiP, and calnexin). The diminished absolute ratio of pseudopodial labeling to the rest of the cell relative to the immunoblot data likely reflects the fact that the pseudopodial domain that penetrates the filter is not limited to the actin-rich portion of the elongated pseudopodial protrusions of MSV-MDCK-INV cells. Indeed, actin was only slightly enriched in the pseudopodial domain by immunoblot (Fig. 4) but significantly enriched (2.3-fold on average) by immunofluorescent quantification.

The limited number of organelle-associated proteins detected in the proteomic analysis represents a strong indication of the purity of the fraction. Indeed, no Golgi, endosomal or lysosomal proteins were identified and few ER and mitochondrial proteins picked up. The major ER protein detected was BiP/GRP78, which is expressed on the cell surface and has recently been identified as a potential target for tumor therapy (9). We were able to detect small amounts of BiP in the pseudopodial fraction by both blot and fluorescence (Figs. 4 and 5) and when compared with the distribution of another ER protein, calnexin, BiP exhibited increased extension to the pseudopodial domain (Fig. 5). Although eight nuclear proteins were identified, the majority are proteins associated with the nuclear pore and involved in nucleocytoplasmic shuttling (i.e. lamin A/C, nucleoporin 93, karyopherin α2, ribonucleoprotein F, Ran, and transportin-SR) (10), whereas SMC1 and other members of the cohesin complex have been localized to the spindle complex during mitosis (11). Interestingly, strong labeling of the nucleoporin complex was detected in the pseudopodial fraction by both blot and fluorescence (Figs. 4 and 5) validating the detection of nuclear pore associated proteins in the proteomic analysis. In the case of pRB1, that has been reported to be predominantly nuclear, enrichment in the pseudopodial fraction relative to the total cell lysate detected by the Kinetworks™ KPSS1.3 phospho-site analysis (Fig. 2B) was confirmed by quantitative immunofluorescence analysis (Fig. 5). Lamin A/C was predominantly nuclear but could also be detected in the pseudopodia (Figs. 4 and 5) and, curiously, was recently found to selectively associate with paxillin in adherent cells (12). For lamin and BiP, their detection in the proteomic analysis therefore reflects not their pseudopodial enrichment but their overall cellular abundance. Nevertheless, limited relative expression does not necessarily exclude a functional role in this cellular domain.

**Rho/ROCK-dependent Pseudopodial Protrusion in MSV-MDCK-INV Cells**—To identify the Met regulators involved in MSV-MDCK-INV cell motility and pseudopod formation, we
treated the cells with specific inhibitors of PI3K (LY294002) and MEK (U0126) identified in the Kinetworks™ screen (Fig. 2) and of ROCK (Y27632) and HSP90 (geldanamycin) identified in the proteomic screen (Table I). Treatment of the cells with U0126 and geldanamycin inhibited MSV-MDCK-INV cell motility and also disrupted MSV-MDCK-INV cell morphology, resulting in enhanced cell spreading (Fig. 6). Geldanamycin-treated cells presented numerous actin stress fibers (Fig. 6F), as previously observed in cells where constitutive HGFR activation was disrupted (5). HSP90 is known to be associated with Met signaling in cancer (13, 14), and treatment of MSV-MDCK-INV cells for 4–6 h with geldanamycin selectively reduced constitutive Met activation (Fig. 6I) confirming a role for HSP90 as a regulator of Met activation in MSV-MDCK-INV cells. Treatment with LY294002 and Y27632 had limited effects on cell motility (Fig. 6A), however inhibition of ROCK with Y27632 altered cell morphology disrupting the expression of the protrusive pseudopodial domains of MSV-MDCK-INV cells and inducing the formation of extended lamellipodia (Fig. 6, G and H).

Consistent with the role of ROCK as a regulator of pseudopodial protrusion, expression of DN-Rho resulted in the expression of highly elongated cells that presented neither actin-rich pseudopodia nor stress fibers (Fig. 7, A–D). In the majority (over 90%) of DN-Rho-transfected cells, the pseudopodial protrusions of MSV-MDCK-INV cells were disrupted, however, in some cells transfected with DN-Rho, myc-tagged DN-Rho could be clearly localized to residual

**Fig. 5. Immunofluorescence localization of proteomics identified proteins to MSV-MDCK-INV pseudopodia.** MSV-MDCK-INV cells transfected with GFP (A and B) and untransfected cells were immunofluorescently labeled for HSP90 (C), calpain-2 (E), vinculin (G), calnexin (I), BiP (K), or EF1α (M), and phalloidin (B, D, F, H, J, L, and N). Alternatively, GFP-actin transfected MSV-MDCK-INV cells (P) were fixed with methanol/acetone and labeled for the nucleoporin complex (NPC; O). Q, the extent of colocalization of the indicated proteins with the actin-rich pseudopodial domain was quantified and is presented relative to labeling in the rest of the cell (± S.E.; *, p < 0.05; **, p < 0.001 relative to GFP). Bar: 20 μm.
actin-rich pseudopodia (Fig. 7, A and B, arrows). Serum-starved MSV-MDCK-INV cells present significantly reduced expression of actin-rich pseudopodia (Fig. 7E), and Rho activation with LPA for 30 min induced the expression of numerous actin densities (Fig. 7, F and H). LPA activation of Rho in the presence of Y27632 resulted in the formation of extended lamellipodial structures (Fig. 7, G and I). ROCK activation therefore regulates the morphology of the pseudopodial protrusions formed in the MSV-MDCK-INV cell line. The high concentration of pseudopodial proteins involved in protein synthesis and degradation is intriguing and argues that the pseudopod is undergoing extensive and dynamic remodeling of its protein composition. Multiple proteins associated with protein translation, including EF1α/H9251, have previously been localized to cellular protrusions (15). Purification of RNA granules from neurons identified 28 proteins associated with mRNA, including EF1α, DDX1, DDX3, and SYNCRIP (16), that were also found in the pseudopod proteome (Table I). We therefore assessed whether RNA is associated with MSV-MDCK-INV pseudopodia by labeling cells with propidium iodide (Fig. 8). In addition to the propidium iodide-labeled nuclei, cytoplasmic PI labeling extended to the actin-rich pseudopodial tips where it colocalized with actin in many, but not all, pseudopodia (Fig. 8A). The cytoplasmic propidium iodide labeling was eliminated upon treatment with RNase indicating that it corresponds to RNA (Fig. 8E). Inhibition of ROCK and formation
of extended lamellipodia resulted in reduced RNA distribution to the pseudopodial domains (Fig. 8C). Quantification showed that ROCK inhibition resulted in a significant reduction of RNA colocalization with pseudopodial actin densities (Fig. 8F). This was not due to volume changes associated with altered morphology of the pseudopodial protrusion, because GFP was still localized to the pseudopodia and indeed exhibited increased colocalization with actin-dense regions upon ROCK inhibition (Fig. 8, B, D, and F).

**DISCUSSION**

The Pseudopod Proteome—We have undertaken the comprehensive analysis of the protein composition of the protrusive pseudopod of the invasive MSV-MDCK-INV cell variant and show it to be a localized signaling domain containing proteins involved in cytoskeleton remodeling, cell adhesion, glycolysis, RNA translocation, and protein translation and degradation. Two recent studies of the proteomic analysis of the pseudopodia of chemotactic-stimulated NIH-3T3 cells (17) and U87 glioma cells (18) used 3 μm pore filters that permit passage of the nucleus; the cells were therefore plated on the filters for a limited time to prevent contamination from the cell body, and a comparative approach was used to identify proteins enriched in the pseudopodia relative to the cell body (17, 18). The pseudopodia of MSV-MDCK-INV selectively pass through 1-μm pore filters that restrict passage of the cell body (6) (Fig. 1), and we chose to identify all detectable proteins, essentially identifying the most abundant pseudopodial proteins irrespective of their enrichment in this domain. Indeed, a number of the proteins identified are not enriched in the pseudopodial fraction yet were localized to this domain by immunofluorescence labeling and blotting (Figs. 4 and 5). One of these, the ER protein BiP whose surface expression is associated with tumor malignancy (9, 19) exhibits increased pseudopodial expression relative to another ER protein calnexin (Fig. 5). Another, lamin, was recently identified as a protein that selectively interacted with paxillin in adherent cells (12). The lack of protein enrichment does not necessarily exclude a functional role in a cellular domain.

Interestingly, although less exhaustive than the current study, almost all the proteins detected in U87 glioma pseudopodia (actin, tubulin, vimentin multiple glycolytic enzymes, annexins I and II, HSP90 and -70, moesin, and tropomyosin) were detected in our study. Both HGF and Met were localized to the pseudopodia of U87 cells, and similarities between the pseudopodial protein complement of the two cell lines may reflect their common autocrine Met activation (5, 18). Actin, filamin, ARP3, tubulin, dynactin, α-actinin, phosphofructokinase, phosphoglucose isomerase, clathrin heavy chain, as well as various ribosomal proteins, tRNA synthetases, and proteasome- and ubiquitin-associated proteins were detected in our analysis as well as that of Lin et al. (17). Discrepancies between the studies with respect to the other pseudopodial proteins identified may reflect the fibroblast origin of NIH-3T3 cells versus the epithelial cell origin of MSV-MDCK-INV cells or differences between chemotactic stimulation of NIH-3T3 fibroblast pseudopodia versus the random, constitutive pseudopodial protrusion of tumor cells. Our ability to generate a highly purified cellular domain and to validate the pseudopodial expression of identified proteins suggests that this analysis will prove to be a useful resource to further understand the complex regulation of pseudopodial protrusion.

**Rho/ROCK Signaling and Tumor Cell Pseudopodial Protrusion**—The pseudopod proteome of MSV-MDCK-INV cells includes the ERM proteins, as well as various myosins, filamin, calpain, calpastatin, Arp2/3, and tropomyosin that are associated with pseudopodial actin dynamics (3). In addition, our screen picked up the coflin inhibitor CAP1 that promotes cofilin and actin turnover (20) and Alix/Aip1 that interact with coflin to promote actin disassembly (21). Furthermore, a major protein detected was Sra-1 that links Rac activation to Arp2/3 and actin assembly via the SCAR/Wave complex (22, 23). Filamin is an actin-cross-linking protein that interacts with various signaling molecules, including Pak1, a target for Cdc42 and Rac1, and regulates remodeling of the cortical actin cytoskeleton (24, 25). The ERM proteins are involved in Rho- and Rac-dependent assembly of focal contacts and actin polymerization (26) and ezrin was previously shown to be present in the pseudopodial domain (6).

MSV-MDCK-INV cells express constitutively active Met (5), and Met-mediated activation of cell motility can occur via activation of either the phosphatidylinositol 3-kinase (27–30) or ERK1/2 MAPK pathways (31). HGF activation of FAK is mediated via Src- and ERK1/2-dependent pathways leading to activation of paxillin and formation of a paxillin-FAK adhesion

![FIG. 8. ROCK-dependent localization of RNA to pseudopodia.](image-url)
complex (31–33). MEK1 inhibition prevented MSV-MDCK-INV cell motility (Fig. 6), and paxillin and FAK are present in the pseudopodial domain (Figs. 4 and 5). Furthermore, phosphorylated Src, MEK1, ERK1/2 (MEK1 substrates), PKBo/Akt1, GSK3α/β (PKBα/Akt1 substrates), and STAT3 (an ERK1/2 substrate), activated downstream of Met/HGF-R (31, 34–37), were all detected in the Kinetworks Tm KPSS 1.3 phospho-site screen of the pseudopodial fraction (Fig. 2). HSP90 is a chaperone known to be associated with Met signaling in cancer (13, 14) and was one of the major proteins picked up in our proteomic screen (Table I). HSP90 was localized to the pseudopodial domain by both immunoblot and immunofluorescence, and the HSP90 inhibitor, geldanamycin, reduced constitutive Met activation and inhibited pseudopod formation (Fig. 6).

In MDCK cells, Met activation leads to activation of Rho, Rac, and Cdc42 (38, 39). Rac activation downstream of Met plays a critical role in HGF-mediated scattering of MDCK cells (38, 40). Dominant active forms of Rho inhibit HGF-induced MDCK cell scattering, whereas dominant negative forms of Rho and inhibitors of ROCK function are dispensable for HGF-mediated lamellipodial spreading and movement (38, 40–42). ROCK-mediated phosphorylation of adducin-α has previously been implicated in ruffling and motility of MDCK cells in response to HGF (43) and phospho-adducin-α is significantly up-regulated in MSV-MDCK-INV pseudopodia (Fig. 2). The demonstration here that Rho stimulates pseudopodial protrusion via ROCK implicates this pathway in Met stimulation of tumor cell motility.

Rho and ROCK have been identified as critical regulators of tumor cell invasivity and metastasis (44, 45), and conditional ROCK activation in colon carcinoma cells stimulates tumor cell dissemination and angiogenesis (46). Rac signaling induces ruffling lamellipodia and the establishment of new substrate contacts, whereas Rho/ROCK signaling stabilizes the adhesions forming focal contacts (47). Focal contact-associated proteins such as FAK, vinculin, paxillin, talin, and α5β1 integrin are localized to MSV-MDCK-INV pseudopodia; however, the cells present few focal contacts (Figs. 1 and 5) suggesting that substrate adhesions in these cells are rapidly turning over. NHE1 is localized to the pseudopodial domain of MSV-MDCK-INV cells where it regulates pseudopodial protrusion and motility (48). Recently, protein kinase A regulation of a Rho/ROCK/p38 MAPK/NHE1 signaling module was characterized in MDA-MB-435 metastatic breast cancer cells (49). Serum deprivation induces pseudopodial elongation and cell invasivity and is associated with inactivation of Rho/ROCK signaling (49). In contrast, serum deprivation of MSV-MDCK-INV cells limits pseudopodial protrusion that can be stimulated by LPA-mediated Rho activation (Fig. 7). Autocrine Met activation and the local signaling context may impact on the role of Rho/ROCK signaling in pseudopodial protrusion. In MDCK cells, oncogenic Ras expression down-regulates Rac leading to up-regulation of Rho and epithelial-mesenchymal transformation (39) supporting a role for the Rho-dependent pseudopodial protrusion described here in the acquisition of motile properties by epithelial derived tumor cells.

RNA Translocation and Local Protein Synthesis—The high concentration of pseudopodial proteins involved in protein synthesis and degradation is intriguing and supports the notion that the pseudopod is undergoing extensive and dynamic remodeling of its protein composition. Met receptor expression is regulated by Cbl-mediated polyubiquitination and proteasome degradation (50, 51), whereas the Cdc42/Rac1-PARG-PKCζ complex responsible for the ubiquitin-mediated degradation of RhoA has recently been shown to regulate cellular protrusive activity and Rho localization to cellular protrusions (52). The proteomic screen also picked up strong expression of the AAA ATPase VCP/p97 (Table I), implicated in a variety of cellular processes, including ubiquitin- and proteasome-dependent processing and degradation (53). Multiple mechanisms that regulate ubiquitin-dependent protein degradation would therefore appear to be present in the pseudopodial domain.

Proteins involved in RNA translocation and protein translation were major components of the pseudopodial fraction representing 22% of all proteins identified (Table I and Fig. 3). Propidium iodide staining of MSV-MDCK-INV cells localized RNA to the pseudopodial domain (Fig. 8), and EF1α was localized to the pseudopodial domain by immunofluorescence and immunoblot (Figs. 4 and 5). Interestingly, RNA binding proteins associate with focal adhesion proteins at early stages of fibroblast cell spreading (12), and paxillin interaction with poly(A)-binding protein 1 (PABP1), a major protein identified in the pseudopod proteome (Table I), regulates nuclear export and delivery to the leading edge of PABP1 and is critical for cell spreading and cell migration (54, 55). Focal adhesion proteins as well as those involved in RNA translocation, protein translation, and nucleocytoplasmic transport represented major protein classes identified in the proteomic analysis of MSV-MDCK-INV pseudopodia (Table I and Fig. 3). The concentration of various focal adhesion proteins (Figs. 4 and 5) together with the absence of stable focal contacts in the pseudopodial domain is highly consistent with a role for the nucleocytoplasmic delivery of mRNA to the pseudopodial domain as a regulator of focal adhesion turnover and dynamics.

ROCK inhibition and the induction of extended lamellipodia were associated with reduced penetration of the actin-rich pseudopodial domain by RNA. β-Actin mRNA is targeted to the leading edge of motile fibroblasts, and serum-mediated signal transduction mechanisms induce the redistribution of β-actin mRNA to the leading edge of fibroblasts (56–58). Interestingly, LPA stimulates β-actin mRNA recruitment to the leading edge of fibroblasts within minutes of addition and acts via activation of Rho/ROCK signaling (59). Expression of EF1α is up-regulated in metastatic mammary carcinoma, a substrate for ROCK, and implicated in the anchoring of β-actin mRNA to the pseudopodial actin cytoskeleton (15, 60). This study identifies Rho/ROCK activation as an inducer of tumor cell pseudopodial protrusions and specifically of RNA translocation to the leading edge of motile cells.

The proteome of purified MSV-MDCK-INV pseudopodia therefore represents a unique, comprehensive analysis that identifies this structure as a dynamic cellular domain where energy production from glycolysis drives cytoskeletal remodeling and protein turnover via local protein synthesis and degradation. By regulating recruitment of mRNA to the pseudopodial domain for local translation, Rho/ROCK signaling may serve to modulate the dynamics and nature of pseudopodial protrusions and thereby promote tumor cell motility and invasion.

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