The adaptor protein Tks5/Fish is required for podosome formation and function, and for the protease-driven invasion of cancer cells

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Summary

Tks5/Fish is a scaffolding protein with five SH3 domains and one PX domain. In Src-transformed cells, Tks5/Fish localizes to podosomes, discrete protrusions of the ventral membrane. We generated Src-transformed cells with reduced Tks5/Fish levels. They no longer formed podosomes, did not degrade gelatin, and were poorly invasive. We detected Tks5/Fish expression in podosomes in invasive cancer cells, as well as in human breast cancer and melanoma samples. Tks5/Fish expression was also required for protease-driven matrigel invasion in human cancer cells. Finally, coexpression of Tks5/Fish and Src in epithelial cells resulted in the appearance of podosomes. Thus, Tks5/Fish appears to be required for podosome formation, for degradation of the extracellular matrix, and for invasion of some cancer cells.

Introduction

To metastasize, tumor cells must invade into tissues and cross tissue boundaries. For most cancer cells, this is thought to require two events: increased motility, and proteolytic degradation of the extracellular matrix (Chang and Werb, 2001; Friedl and Wolf, 2003). The motility of cancer cells is driven by reorganization of the cytoskeleton and of contacts between the cell and the matrix. This involves many of the same proteins that are required for normal cell motility, including members of the Rho family of small GTPases, and actin-associated proteins and regulators (Ridley et al., 2003). Degradation of the extracellular matrix occurs via the concerted action of a number of proteases, including matrix metalloproteases (MMPs), cathepsins, and serine proteases such as seprase and uPA (Chen, 1992; Bauvois, 2004). The secretion of these proteases by tumor cells results in the generation of "migration tracks" in the extracellular matrix, through which the cells can move. The relevance of proteases to the invasive capacity of most tumor cells is supported by in vitro assays where invasion is blocked by protease inhibitors, as well as in mouse model systems (Chang and Werb, 2001).

The transformation of rodent and chicken fibroblasts by activated forms of the protein tyrosine kinase Src has long provided a model for studying the properties of cancer cells (Martin, 2001). Src-transformed cells are not only morphologically transformed, but are also highly invasive. They constitutively produce uPA and MMPs (Bell et al., 1990; Chen et al., 1991). They can degrade thin films of extracellular matrix proteins such as fibronectin, laminin, and collagen (Chen et al., 1984, 1985). And they also are able to invade through matrigel (a mixture of basement membrane proteins) in a Boyden chamber assay. How is the activation of these extracellular proteases coordinated? Many studies have implicated specialized structures called podosomes, which are also often referred to as rosettes or invadopodia. Podosomes were first described as electron-dense protrusions of the ventral membrane of Src-transformed cells (David-Pfeuty and Singer, 1980; Tarone et al., 1985). They were subsequently shown to have a diameter of approximately 0.4 μm, to extend from the cell body into the matrix layer, to be enriched in actin, to be highly dynamic structures with a turnover time in the order of minutes, and to cluster membrane proteases (Chen, 1989, 1990). Following the discovery of podosomes in Src-transformed cells, similar structures were identified in osteoclasts, macrophages, and dendritic cells, all terminally differentiated cells of the monocyte

SIGNIFICANCE

Podosomes, or invadopodia, were first described as electron-dense structures on the ventral surface of Src-transformed fibroblasts, where they form the attachment points between cell and substratum. These structures are also found in normal cells such as osteoclasts and macrophages, and in some invasive cancer cells. Podosomes are thought to promote the invasive properties of cells, by clustering proteases involved in extracellular matrix degradation. We have previously shown that the scaffolding protein and Src substrate Tks5/Fish localizes to podosomes. We now show that Tks5/Fish is required for podosome formation, and for matrix degradation and invasion. These findings support a broader investigation of Tks5/Fish and its binding proteins both as markers of invasive disease and as potential therapeutic targets.

lineage with the ability to cross tissue boundaries (Linder and Aepfelbacher, 2003; Buccione et al., 2004). More recently, certain invasive human cancer cells, particularly breast cancers and melanomas, were also shown to contain podosomes (Kelly et al., 1994; Monsky et al., 1994; Bowden et al., 1999). The cell types in which podosomes are found support the notion that these structures are involved not just in cell motility, but also in extracellular matrix degradation and invasiveness.

Podosomes are actin-rich structures, and contain many actin regulatory proteins, such as WASp, Arp2/3, and the small G proteins cdc42 or RhoA, as well as actin-associated proteins such as gelsolin. Podosomes also contain proteins that are commonly found in focal adhesions, such as focal adhesion kinase (FAK), integrin receptors, paxillin, Src, and vinculin. However, other proteins found in podosomes are not found in focal adhesions. These include the actin-associated protein cortactin, the GTPase dynamin, the proteases seprase and MT1-MMP (also known as MMP14), and the scaffolding protein Tks5/Fish (for review, see Linder and Aepfelbacher, 2003; Buccione et al., 2004). Several studies have shown that actin turnover is important in the formation of podosomes in various cell types. For example, WASp, Arp2/3, cdc42, RhoA, and gelsolin are all required for podosome formation, as is dynamin (Linder et al., 1999, 2000; Chellaiah et al., 2000a, 2000b; Ochoa et al., 2000; Mizutani et al., 2002; Moreau et al., 2003; Berdeaux et al., 2004). Furthermore, it was shown in a melanoma cell line that efficient extracellular matrix degradation required the podosomal localization of MT1-MMP (Nakahara et al., 1997). The mechanisms by which podosome formation and function are regulated are therefore of interest in the context of tumor metastasis.

We recently used a novel Src substrate screening assay to isolate a new scaffolding protein with an aminoterminal phox homology (PX) domain, and five SH3 domains (Lock et al., 1998). We originally called this protein Fish. However, because of the difficulties associated with accessing the relevant literature and databases using the name Fish, we now propose to revert to the original clone id name, Tks5. To avoid confusion during this transition, we will refer to the protein as Tks5/Fish throughout this manuscript.

We demonstrated that Tks5/Fish was cytoplasmic in normal fibroblasts, but was relocalized to podosomes in cells transformed by Src (Abram et al., 2003). The PX domain of Tks5/Fish is both necessary and sufficient for podosome localization. Like most, if not all, PX domains, the PX domain of Tks5/Fish associates with phosphatidylinositol lipids, with preference in vitro for PI 3-P and PI 3,4-P_2. The podosomes of Src-transformed cells are not enriched in PI 3-P (unpublished data), suggesting that PI 3,4-P_2 may be responsible for targeting Tks5/Fish to podosomes, but this remains to be established. The binding partners for most of the SH3 domains of Tks5/Fish are yet to be uncovered. However, we have shown that the 5th SH3 domain associates with members of the ADAMs family of metalloproteases. Further analysis showed that as a consequence of this interaction, ADAM12, which is usually associated with intracellular membranes in fibroblasts, is found in the podosomes of Src-transformed cells (Abram et al., 2003).

We hypothesized that Tks5/Fish might be involved in the formation and/or function of podosomes. Here, we describe the results of experiments designed to test this hypothesis, in both Src-transformed fibroblasts and in human cancer cells.

Results

Reduction of Tks5/Fish expression in Src-transformed cells alters their morphology

We utilized short hairpin (sh) RNA technology to reduce the level of Tks5/Fish in Src-transformed cells. We first tested the ability of four short interfering (si) RNAs targeted to different regions of the *Tks5/Fish* gene to reduce expression of Tks5/Fish in fibroblasts. Two of these siRNAs, 1 and 4, were effective (data not shown). We then went on to create shRNA expression vectors containing these sequences, transfected them into NIH3T3 (3T3) cells transformed with the activated cSrc allele Y527F (Src3T3), selected several drug-resistant clones, and then tested for Tks5/Fish expression by immunoblotting. Only shRNA sequence 4 efficiently reduced the expression of Tks5/Fish when expressed in this hairpin vector rather than as a short RNA (data not shown). We chose three knockdown clones that had reduced Tks5/Fish expression (4.20, 4.21, and 4.24), as well as three control clones that were drug selected after transfection with a vector containing a control sequence designed not to target murine sequences (C1, C2, and C3), for further analysis. Immunoblotting confirmed an approximately 5-fold reduction in Tks5/Fish levels in the knockdown, but not control, clones (Figure 1A, top). An irrelevant protein of approximately the same size (the PDGF receptor) was unaffected by the shRNA (data not shown), and all cells retained expression of the activated Src that was used to transform them (Figure 1A, bottom). We also determined that the overall level of tyrosine phosphorylation was unchanged when compared to Src3T3 cells (Figure 1B), as was the ability of the cells to secrete the metalloproteases MMP2 and MMP9 (Figure 1C). We noticed no obvious differences in the growth rates of the control and knockdown clones (data not shown). Furthermore, the knockdown clones were still able to form foci when cocultured with normal fibroblasts, although we did notice that they displayed a fusiform morphology, rather than the more rounded appearance of the control clones (data not shown).

We next examined more closely the morphology of the knockdown and control clones, by plating them onto coverslips and visualizing F actin with fluorescent phalloidin (Figure 2A). The control clones retained the morphology of the parental Src3T3 cells, with few actin fibers and many podosomes. In contrast, the knockdown clones were larger and flatter, with some actin stress fibers, but few obvious podosomes. The morphological changes observed were present in essentially all cells in the population. Since only one of our shRNA vectors efficiently reduced Tks5/Fish expression, we were concerned that the differences we observed might be due to off-target effects of the interfering RNA. We therefore wanted to test whether the phenotypic differences observed could be reversed by reintroduction of the Tks5/Fish protein. We took advantage of the fact that only 15 of the 19 base pairs encompassing siRNA 4 are conserved in the human *Tks5/Fish* cDNA, and that, as determined by transfection, the levels of the human Tks5/Fish protein were unaffected by siRNA 4 (data not shown). We microinjected 4.20 cells with an expression vector containing a cDNA encoding human Tks5/Fish, incubated the cells for 24 hr, and then stained the cells with fluorescent phalloidin and with an antibody that recognizes Tks5/Fish. We observed that all microinjected cells were replete with podosomes, compared to the uninjected cells in the same field,

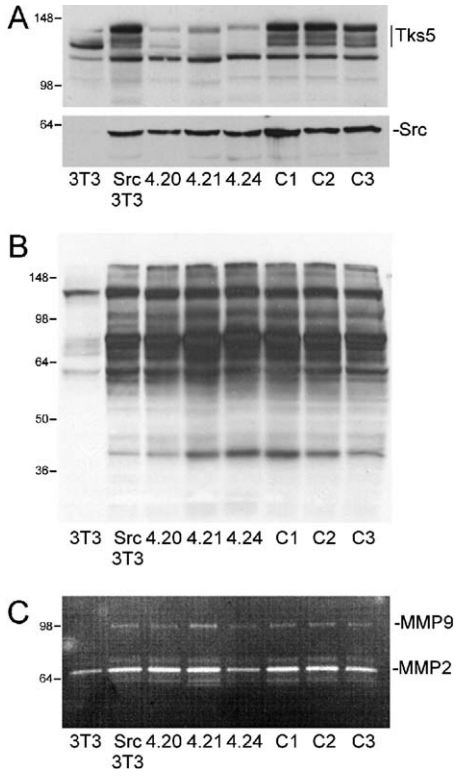

Figure 1. Generation of clones of Src-transformed cells with reduced Tks5/Fish expression

Parental NIH3T3 (3T3) and Src-transformed NIH3T3 (Src3T3) cells, as well as control (C1, C2, and C3) and Tks5/Fish knockdown (4.20, 4.21, and 4.24) clones were grown to the same density and lysed, and protein concentrations determined and normalized. The comparative levels of Tks5/Fish and Src (**A**) as well as total phosphotyrosine content (**B**) were determined by immunoblot. In fibroblasts, Tks5/Fish presents as a closely spaced triplet of bands, as shown. Conditioned media from cells grown to the same density were collected and assayed for gelatinase activity by zymography (**C**). Molecular weight markers are shown on the left side of each panel.

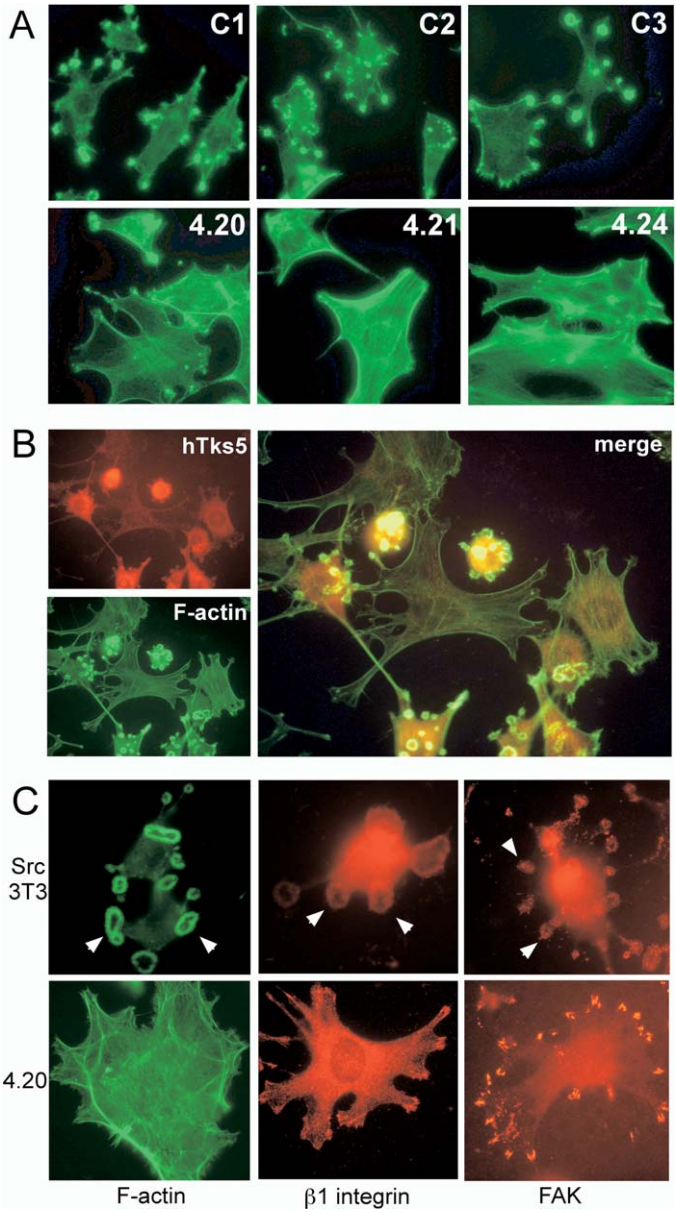

Figure 2. The impact of Tks5/Fish protein on the morphology of Src-transformed cells

A: F actin morphology was determined in control (C1, C2, and C3) and Tks5/Fish knockdown (4.20, 4.21, and 4.24) clones 48 hr after plating on glass coverslips, by fixation and staining with phalloidin.

B: Tks5/Fish knockdown 4.20 cells were plated on glass coverslips, then microinjected with an expression vector encoding human Tks5/Fish (hTks5). Twenty-four hours later, the cells were fixed and stained with antibodies to Tks5/Fish to visualize the microinjected cells, and phalloidin to visualize F actin. Tks5/Fish staining is in red, and F actin staining in green.

C: Parental Src3T3 cells and the Tks5/Fish knockdown clone 4.20 were grown on glass coverslips, then fixed and stained with either phalloidin, or antibodies specific for β1 integrin or FAK, as shown. For each Src3T3 cell image, arrowheads mark the positions of two typical rosettes of podosomes.

which retained their more flattened morphology (Figure 2B). We therefore conclude that the Tks5/Fish protein is rate-limiting for the formation of podosomes in Src3T3 cells.

We chose one knockdown (4.20) clone for further analysis, comparing it to the Src3T3 cells. For each staining condition, one representative cell is shown in Figure 2C. In the parental cells, all of the F actin staining was concentrated in the podosomes, which were present in characteristic rosettes. In contrast, podosomes were not observed in the 4.20 cells. Instead, actin stress fibers and cortical actin staining were observed. Some of the peripheral actin appeared to be present in an "arrowhead" type of configuration, similar to that found in focal adhesions. To analyze this further, we stained cells with antibodies to β1 integrin and FAK, proteins which are found in focal adhesions in normal cells (Parsons, 2003). We found that a proportion of these proteins were localized to podosomes in the parental cells, consistent with the reports of others (Linder and Aepfelbacher, 2003). In contrast, in the 4.20 cells, these proteins were localized to discrete spots in the periphery, coincident with the concentrations of F actin (Figure 2C, and data not shown), which may therefore represent focal adhesions.

Src-transformed cells with reduced Tks5/Fish expression show defects in matrix degradation and invasion

We next tested whether a reduction in the level of Tks5/Fish protein influenced the ability of the cells to degrade extracellu-

lar matrix proteins. To do this, we took advantage of an assay in which cells are plated onto fluorescently labeled matrix proteins (in this case FITC-gelatin), and degradation is assessed microscopically (Bowden et al., 2001). In this assay, 3T3 cells do not degrade the gelatin, whereas the Src3T3 efficiently degrade the matrix layer in discrete spots coincident with the presence of podosomes, as described by others previously (data not shown). We observed that the control clones retained their ability to degrade gelatin, but the knockdown clones were unable to degrade the matrix protein efficiently during the course of this assay (Figure 3A). We next tested whether expression of human Tks5/Fish could rescue matrix degradation (Figure 3B). We found areas of degradation underneath the microinjected cells, but not around cells in another area of the coverslip that was not microinjected. We conclude therefore that matrix degradation requires Tks5/Fish.

In vitro, the invasive capacity of cells can be measured by testing their ability to invade through a layer of matrigel placed in a Boyden chamber. We next tested our clones in this assay (Figure 3C). Src-transformed cells are markedly more invasive than the parental fibroblasts from which they derive. All the control clones retained this degree of invasiveness. However, the Tks5/Fish knockdown clones behaved more like the 3T3 cells, and exhibited a marked reduction in invasive capacity. We also tested the motility of the knockdown clones (Figure 3D). Only one clone showed a statistically significant reduction in migration; the other two clones did not differ significantly from the parental cells or from the control clones. These data suggest that Tks5/Fish is not required for the enhanced motility of Src-transformed cells.

From these experiments, we conclude that Tks5/Fish is required for the formation of podosomes, as well as for matrix degradation and in vitro invasiveness.

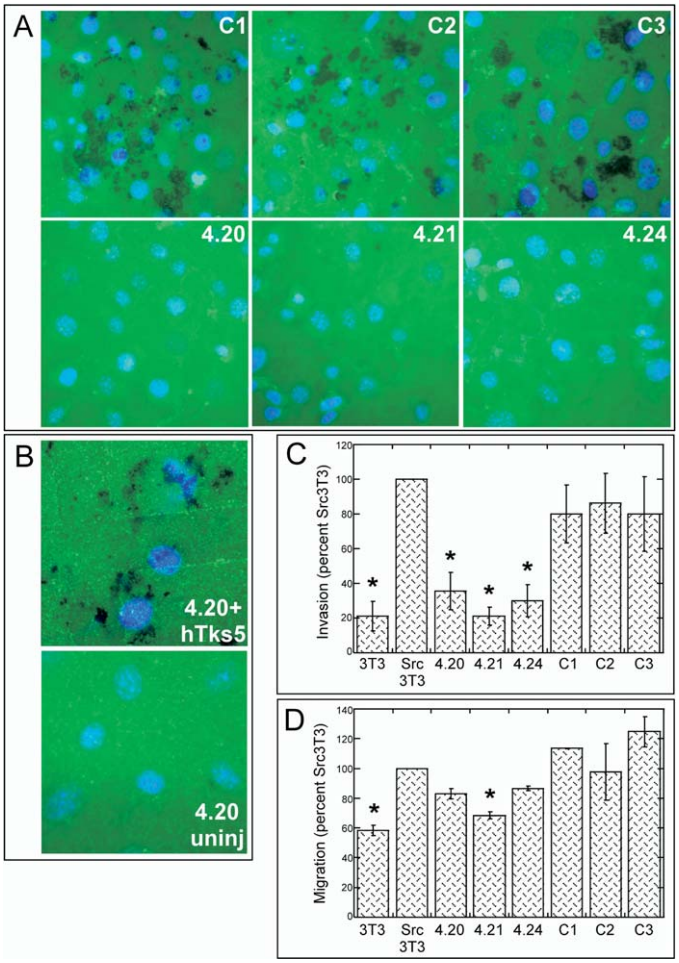

Figure 3. Analysis of the invasive capacity of control and Tks5/Fish knockdown cells

A: Control (C1, C2, and C3) and Tks5/Fish knockdown (4.20, 4.21, and 4.24) clones were tested over a period of 48 hr for the ability to degrade a thin layer of FITC-gelatin coated onto glass coverslips. Degradation is indicated by the dark patches (holes) within the fluorescent monolayer. Cell density is indicated by Hoechst staining of nuclei (blue).

B: Tks5/Fish knockdown 4.20 cells were plated on FITC-gelatin coated glass coverslips, then microinjected with an expression vector encoding human Tks5/Fish (hTks5). Twenty-four hours later, the cells were fixed and stained with antibodies to Tks5/Fish to visualize the microinjected cells (not shown), and Hoechst to visualize nuclei (blue). The top panel shows three microinjected cells and surrounding patches of gelatin degradation. The bottom panel shows a region of the coverslip containing uninjected (uninj) cells.

C: 3T3 and Src3T3, along with control (C1, C2, and C3) and Tks5/Fish knockdown (4.20, 4.21, and 4.24) clones, were tested for their ability to invade through a thin layer of matrigel in a Boyden chamber assay over a period of 24 hr. The data were compiled from three independent experiments. All measurements were calculated as percent of control (Src3T3) and error bars calculated as propagated standard errors of the mean of triplicate measurements from each experiment. Statistical significance was determined by paired t tests with an asterisk indicating a statistical difference from the control ($p < 0.05$).

D: 3T3 and Src3T3, along with control (C1, C2, and C3) and Tks5/Fish knockdown (4.20, 4.21, and 4.24) clones, were tested for migration in a Boyden chamber assay over a period of 24 hr. Data were plotted and analyzed as described in **C**.

Many human cancer cells express Tks5/Fish

Podosomes have been described in several human cancer cell lines, particularly invasive breast carcinomas and melanomas, and their presence has been correlated with invasiveness in vitro (Monsky et al., 1994; Kelly et al., 1998; Bowden et al., 1999). To determine whether Tks5/Fish might play a role in human cancer cells, we first measured the levels of Tks5/Fish protein in a number of cancer cell lines (Figure 4A). We first compared two poorly invasive breast cancers, T47D and MCF7, with the more invasive lines Hs578t, MDA-MB-231, and Bt549 (Thompson et al., 1992; Bowden et al., 1999). Tks5/Fish was expressed at very low levels in T47D and MCF7 cells, visible only at longer exposures of the blot (data not shown), but was more highly expressed in the invasive cells. All the melanoma cell lines we tested (including the RPMI-7951 and C8161.9 lines used later, as well as several others, had Tks5/Fish expression levels as high or higher than Bt549 cells; data not shown). As in fibroblasts, Tks5/Fish is expressed in multiple forms in these cells; it is possible, but remains to be established, that alternative splicing and/or phosphorylation plays a role in the generation of these different isoforms. Interestingly, when we compared the levels of *Tks5/Fish* mRNA by RT-PCR, we detected very low levels of mRNA in the T47D cells, but the MCF7 cells contained approximately the same level of mRNA as the invasive lines (Figure 4B), suggesting that Tks5/Fish expression is likely to be controlled at both the RNA and the protein level.

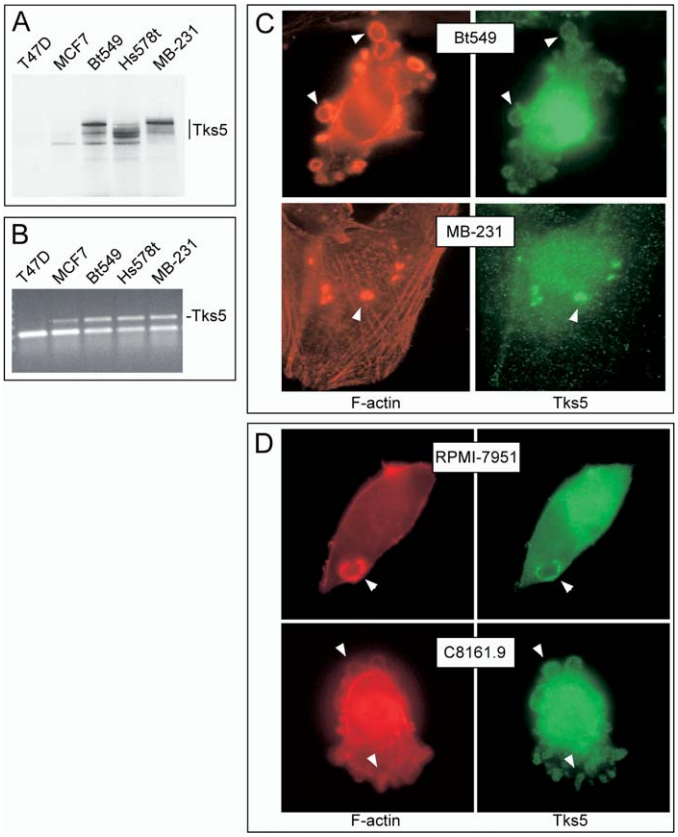

Figure 4. The expression and localization of Tks5/Fish in human cancer cell lines, and in tumor samples

A: Whole-cell lysates derived from the indicated breast carcinoma cell lines were normalized for protein concentration, then immunoprecipitated and immunoblotted with a Tks5/Fish antibody. Tks5/Fish is present as a closely spaced series of bands, present in different ratios depending on the cell line tested, as shown.

B: RT-PCR was used to make a semiquantitative comparison of Tks5/Fish mRNA levels (upper band) across the indicated breast carcinoma cell lines. The lower band is nonspecific.

C: Breast carcinoma cells were grown on glass coverslips for 48 hr, fixed, and stained with antibodies for Tks5/Fish, and phalloidin to visualize F actin, as shown. In each image, the localization of representative rosettes of podosomes is marked by arrowheads.

D: The melanoma cell lines RPMI-7951 and C8161.9 were grown and processed as described in **C**. Rosettes of podosomes are marked by arrowheads.

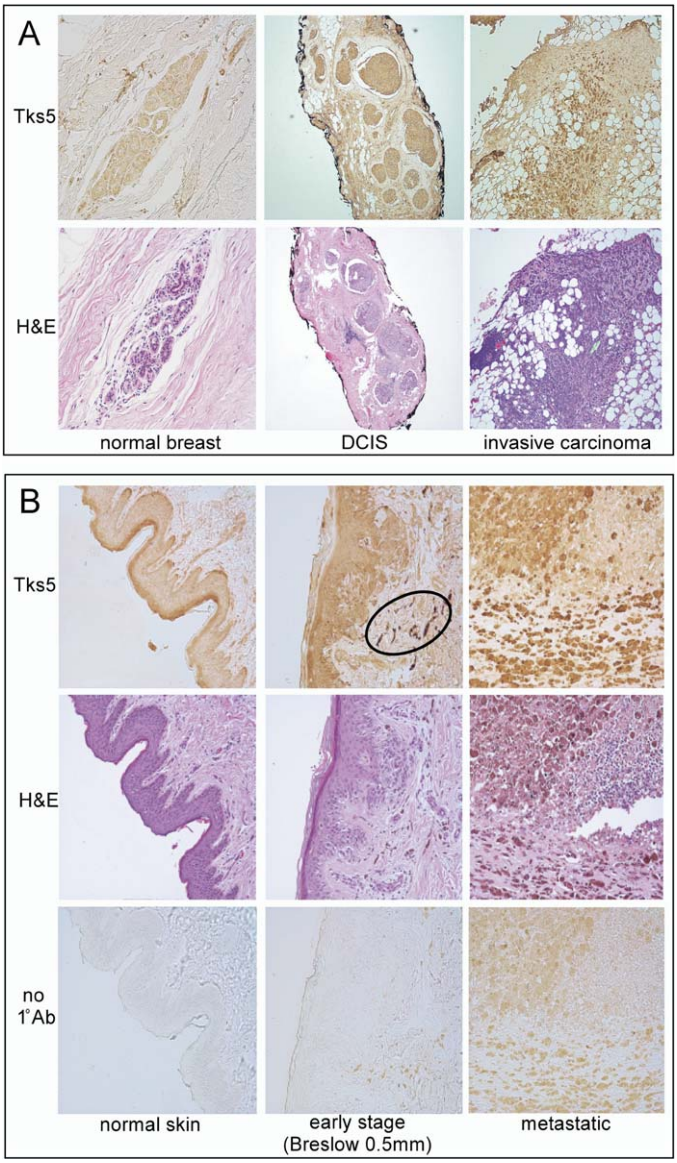

Figure 5. The expression and localization of Tks5/Fish in human tumor samples

Histological samples of normal and cancerous breast (**A**) and skin (**B**) tissue were stained with Tks5/Fish antibodies or with hematoxylin and eosin (H&E), as shown. The oval in **B** shows high levels of Tks5/Fish in invading tumor cells. Background staining from the secondary antibody is also indicated in **B**. DCIS, ductal carcinoma in situ.

We next determined whether Tks5/Fish was present in the podosomes of human cancer cells (Figures 4C and 4D). We should note that unlike Src-transformed fibroblasts, where podosomes are found in the majority of cells, in our hands, only a small percentage of the breast carcinoma and melanoma cells have podosomes at any given time. However, we did find that when we detected podosomes in both breast cancer cell lines (Bt549 and MDA-MB-231) and melanomas (RPMI-7951 and C8161.9), Tks5/Fish appeared to be colocalized to these structures, albeit it at very low levels.

To determine whether Tks5/Fish could also be detected in human tumor samples, we first used immunohistochemistry to visualize Tks5/Fish in samples of normal breast and breast cancer (Figure 5A). We noted moderate Tks5/Fish expression that was restricted to ductal cells in normal breast tissue. However, both ductal carcinoma in situ (DCIS) and metastatic breast cancer samples showed extensive Tks5/Fish expression in the malignant cells. No staining was observed with preimmune rabbit serum as the primary, and a second Tks5/Fish antibody that recognizes a different epitope gave the same staining pattern (data not shown). In total, we have analyzed 14 samples of human breast tissue (4 normal, 3 DCIS, 3 microinvasive, and 4 metastatic); all the precancerous and tumor cells had higher Tks5/Fish staining than the surrounding normal tissue. We next went on to examine samples of skin tissue

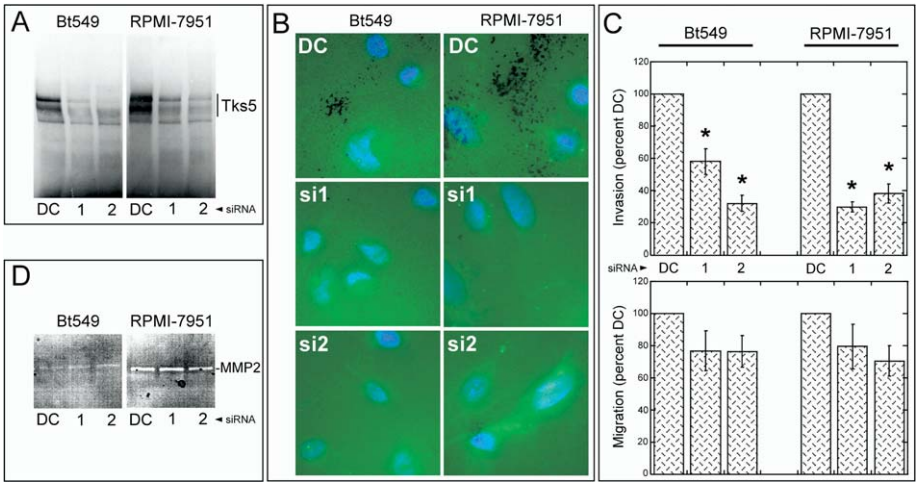

Figure 6. Generation and analysis of invasion in human cancer cells with reduced Tks5/Fish expression

Two siRNAs (si1 and si2) targeting human Tks5/Fish, or a pool of duplex controls (DC), were transiently transfected into BT549 breast carcinoma cells or RPMI-7951 melanoma cells. Thirty hours posttransfection, the cells were either processed for the measurement of Tks5/Fish protein levels following immunoprecipitation of lysates with a Tks5/Fish antibody (**A**), plated onto FITC-gelatin coated glass coverslips to measure gelatin degradation activity (**B**), transferred to Boyden chambers to measure migration or invasion through matrigel (**C**), or quiesced for 48 hr in order to test the conditioned medium for gelatinase activity by zymography (**D**). The data in each panel derive from separate transfections, but in each case, immunoblotting was conducted to ensure that Tks5/Fish levels were reduced by the siRNA treatment. The data in **C** represent the mean of triplicate measurements (with propagated standard errors) expressed as a percentage of the duplex control treated cells from a single, representative experiment. Asterisks represent a statistical difference from the control ($p < 0.05$).

(Figure 5B). We detected moderate Tks5/Fish staining in the basal layer, but higher staining in both an early stage melanoma and a metastatic tumor. Interestingly, there was particularly intense staining in those melanoma cells that were invading into the surrounding tissue from the early stage melanoma (middle panels). In total, we have analyzed 16 skin samples (4 normal, 7 with Breslow grading from 1–4 mm, and 4 metastatic), and have always observed higher Tks5/Fish staining in the tumors. We therefore conclude that Tks5/Fish is expressed in human tumor cells in vivo.

Tks5/Fish is required for invasion of human cancer cells

These findings prompted us to test whether Tks5/Fish was required for invasiveness in human cancer cells. We first chose one breast cancer cell line (Bt549) and one melanoma (RPMI-7951) for our analyses. We transfected them with two independent siRNAs targeting Tks5/Fish, or with a mixture of siRNAs designed to not reduce expression of cell proteins (duplex control). Reduction of all isoforms of Tks5/Fish with the specific siRNAs was confirmed by immunoblot analysis (Figure 6A). We next tested the ability of these cells to degrade thin films of gelatin (Figure 6B), and found that in both cases, the Tks5/Fish siRNA transfected cells showed reduced degradation compared to controls. We viewed at least 25 fields, and counted as positive all fields that contained any degradation, however small. For both cell lines, essentially all fields of duplex control transfected cells showed degradation. However, degradation was only observed in 55% or fewer of all fields of Tks5/Fish knockdown cells. Furthermore, even when degradation was observed, it was much less extensive in the Tks5/Fish knockdowns than in the controls (compare for example DC and si2 for RPMI-7951 in Figure 6B). We next tested the invasiveness of the siRNA-transfected cells in a Boyden chamber assay (Figure 6C). In each case, we noted a significant, but not absolute, reduction in matrigel invasion in the cells with reduced Tks5/Fish expression. No significant differences in motility were observed. We also found a similar requirement for Tks5/Fish for invasiveness of the other tumor cell lines (Figure 7). As was the case for the Src-transformed cells with reduced Tks5/Fish expression, no significant differences in MMP secretion were observed (Figure 6D). These data suggest that Tks5/Fish is also required for optimal matrix degradation and invasiveness of human cancer cells.

It has recently been noted that cancer cells can penetrate extracellular matrix either in a manner that requires proteases, or using a protease-independent mechanism that, in some cases at least, might be due to amoeboid-type movement (Sahai and Marshall, 2003; Wolf et al., 2003). To determine which mechanism might be in force in the cells we are studying, we

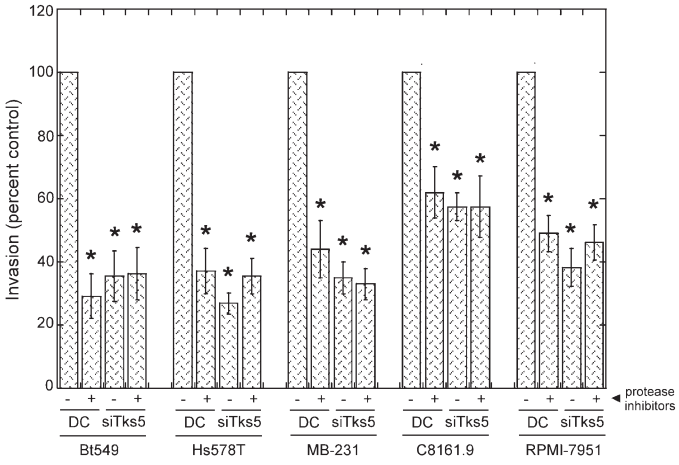

Figure 7. Tks5/Fish is required for protease-dependent invasion

siRNA-transfected breast carcinoma (BT549, MB-231, Hs578t) and melanoma (C8161.9, RPMI-7951) cells were transfected prior to invasion assays as described in Figure 6, except that a cocktail of protease inhibitors (or vehicle alone) were added to each experimental group of cells in order to monitor the protease dependence of invasion through matrigel. The invasion of each experimental group of cells is expressed as a percentage of the duplex control-treated cells that lacked protease inhibitors in the medium, and was analyzed as described in Figure 6. siTks5 refers to the use of either siRNA2 alone (RPMI-7951; refer to Figure 6), a combination of siRNA1 and 2 (BT549, MB-231), or a pool of siRNAs (Hs578T, C8161.9). All of these conditions reduce Tks5/Fish protein levels.

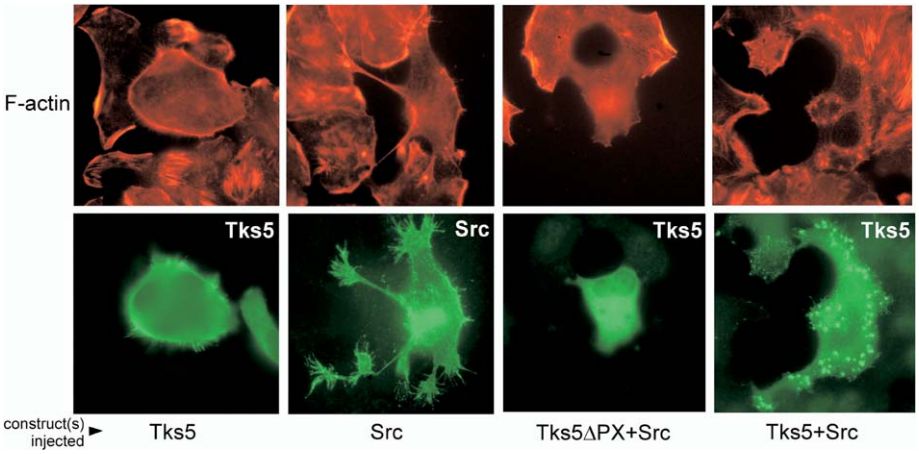

Figure 8. The generation of podosomes in T47D cells

T47D cells were microinjected with the indicated expression vectors encoding either wild-type Tks5/Fish, Tks5/FishΔPX, and/or Src. Twenty-four hours later, the cells were fixed and stained with antibodies to Tks5/Fish or Src, and phalloidin to visualize F actin, as indicated in each panel.

repeated the invasion experiments in the presence or absence of a cocktail of protease inhibitors that have previously been shown to block proteolytic invasion (Sahai and Marshall, 2003). We found that whereas the invasion of the duplex control-treated cells could be partially blocked by the protease inhibitors, little further reduction in invasion of the Tks5/Fish siRNA treated cells was detected in the presence of protease inhibitors (Figure 7). These data suggest that Tks5/Fish is required for the protease-driven invasion of matrigel.

Expression of Tks5/Fish in T47D cells results in the generation of podosomes

Our studies to date have determined that the Tks5/Fish protein is required for the formation of podosomes. We next wanted to determine whether introduction of Tks5/Fish into cells that do not normally express it was sufficient to drive podosome formation. We chose T47D cells, which have very low levels of both Tks5/Fish mRNA and protein. When these cells were injected with an expression vector encoding Tks5/Fish, some morphological changes in the actin cytoskeleton were noted, but no podosomes were observed (Figure 8). However, when Tks5/Fish was coinjected with an activated form of Src, punctate actin-rich structures containing Tks5/Fish were evident on the ventral surface of the cell. No such structures were seen when Src was expressed alone, although activated Src did have profound effects on both morphology and the actin cytoskeleton. Consistent with our previous findings that the PX domain of Tks5/Fish is required to target it to podosomes (Abram et al., 2003), when Tks5/FishΔPX and Src were coexpressed, no podosomes were observed. Interestingly, in the presence of Tks5/FishΔPX, expression of activated Src did not result in any gross morphological changes; rather, all actin polymerization in the cell appeared to be inhibited. We conclude that Tks5/Fish, when expressed in cells containing active Src, is able to promote the formation of podosomes.

Discussion

Tks5/Fish is a large adaptor protein and Src substrate that we have previously shown to be present in the podosomes of Src-transformed cells (Abram et al., 2003). We have now used shRNA technology to demonstrate that the Tks5/Fish protein is required for the formation of podosomes in Src-transformed cells. In the absence of robust Tks5/Fish expression, Src-transformed fibroblasts do not revert to an untransformed phenotype, but they do adopt a larger, flatter morphology, with some normal actin cytoskeleton. In addition, while the parental Src-transformed cells attach to the substratum via the podosomes, the knockdown cells have ventral structures that resemble focal adhesions, containing integrins, FAK, and vinculin (Figure 2C and data not shown). This might suggest that, when present, the Tks5/Fish protein recruits focal adhesion proteins such as these, as well as cortical proteins such as cortactin, into the podosomes.

Our data show that Tks5/Fish is required for podosome formation. We also show that the introduction of Tks5/Fish (along with activated Src) results in the formation of podosomes in cells that do not normally have them. We have succeeded in generating podosomes in T47D cells (Figure 8), as well as MCF7 and 293 cells (data not shown), all epithelial cells with a very low level of endogenous Tks5/Fish protein. We have previously shown that the PX domain of Tks5/Fish is required to target the protein to preformed podosomes in Src-transformed cells (Abram et al., 2003). In keeping with this, a Tks5/Fish protein lacking the PX domain was unable to promote podosome formation when coinjected with activated Src into T47D cells. The PX domain of Tks5/Fish is a phosphoinositide binding domain, with an apparent preference for PI3-P and PI3,4-P_2. The model that we are currently testing is that Tks5/Fish is first recruited diffusely to membranes via the interaction of its PX domain with a phosphatidylinositol lipid (perhaps PI3,4-P_2), and then a clustering of Tks5/Fish and its associated proteins results in the formation of podosomes. Perhaps one of the functions of activated Src in this assay is to (indirectly) generate the lipid product that recruits Tks5/Fish to the plasma membrane. It is also possible that Tks5/Fish itself, as well as other podosome proteins, need to be tyrosine phosphorylated for podosome formation. Indeed, it has been observed previously that podosomes are enriched in tyrosine phosphorylated proteins (Mueller et al., 1992). The microinjection assay we describe here will now allow us to dissect the roles of Src, and the different domains of Tks5/Fish, in podosome formation.

Why is Tks5/Fish needed for podosome formation? One model would be that it associates with, and thus recruits, pro-

teins known to be required for podosome formation. In keeping with this model, we have preliminary evidence that the 3rd SH3 domain of Tks5/Fish is able to associate with the actin regulatory protein N-WASp (unpublished data); N-WASp and the related protein WASp are essential for podosome formation in Src-transformed fibroblasts and macrophages, respectively (Linder et al., 1999; Mizutani et al., 2002).

Analysis of Src-transformed cells with reduced Tks5/Fish expression revealed that they are markedly handicapped in both the ability to degrade a thin film of gelatin, and in invasion through matrigel, both properties that are thought to involve proteases, particularly matrix metalloproteases. In this regard, it is interesting to note that the knockdown cells still secrete MMP2 and MMP9, in both precursor and cleaved forms. At this point, it is not clear why these secreted MMPs are not able to degrade the extracellular matrix. Perhaps they are required to be locally activated at podosomes to be functional. In keeping with this, the podosome localization of MT1-MMP, an activator of MMP2 and MMP9, appears to be required for matrix degradation (Nakahara et al., 1997).

Epithelial cells typically express low levels of the Tks5/Fish protein. However, we have noted increased Tks5/Fish expression in many cancer cell lines, including invasive breast carcinomas (Figure 4) and melanomas (data not shown). This finding was not restricted to cells in culture; we also noted marked staining with Tks5/Fish antibodies in paraffin embedded tumor samples (Figure 5). We are currently extending these analyses to more cell lines and tissues, in order to address whether Tks5/Fish levels may be predictive of invasive disease. This would be particularly useful in melanoma, where there are few markers of metastatic disease. One breast cancer cell line with low Tks5/Fish levels (T47D) has barely detectable *Tks5/Fish* mRNA, whereas the other (MCF7) has mRNA levels similar to those in the invasive lines. These findings suggest that Tks5/Fish is regulated at both the RNA and the protein level, and that microarray analysis will not always be suitable to measure Tks5/Fish levels in tumor samples. We have also observed that the half-life of the Tks5/Fish protein is considerably longer in Src-transformed cells than in their normal counterparts (unpublished data), which might suggest that tyrosine phosphorylation, or podosomal localization, enhances its stability. More work is clearly needed to determine how the level of the Tks5/Fish protein is controlled.

Invasive breast cancer cells and melanomas such as those used in this study have been previously reported to have podosomes (Kelly et al., 1994; Monsky et al., 1994; Bowden et al., 1999). We have also observed podosomes in these cells, although we find that in a given population of cells at a given time, only the minority will have obvious podosomes. Perhaps podosomes only form at a particular point in the cell cycle, or in response to a particular signal. This has previously been observed in cells of the macrophage/monocyte lineage. For example, podosomes form in osteoclasts as they differentiate (Destaing et al., 2003), and in dendritic cells as they initiate migration in response to a stimulus such a lipopolysaccharide (Burns et al., 2004). It is also known that podosomes have a rapid turnover. In the future it will be important to determine the signals that mediate the formation and turnover of podosomes of tumor cells; this control point might represent an attractive target for therapeutic intervention. Regardless, in the invasive breast cancer cells and melanomas where podosomes were observed, we detected Tks5/Fish colocalization with the structures, suggesting that Tks5/Fish is a common component of podosomes.

To determine whether Tks5/Fish is also required for the invasive properties of human cancer cells, we used transient transfection of siRNAs, resulting in a reduction of Tks5/Fish expression of approximately 80%. We noticed a significant reduction in the ability of such cells to invade matrigel, compared to duplex control cells. However, we noted that in each case, the inhibition was not complete. Furthermore, invasion of the control cells was partially inhibited by treatment with a cocktail of protease inhibitors, whereas the Tks5/Fish knockdown cells were not further inhibited by the addition of protease inhibitors to the assay. This suggests that some of the cells in the population can use a protease-independent method of invasion, as recently described. Interestingly, it has been shown that when proteolytic activity is inhibited, MDA-MB-231 cells use an amoeboid movement to penetrate matrigel (Wolf et al., 2003). Our data support the hypothesis that Tks5/Fish is required for proteolytic invasion. It will be interesting to determine in the future how many cancer cells use proteolytic mechanisms to invade, and whether such an invasive strategy correlates with the presence of podosomes in the cells.

In conclusion, we have shown that the adaptor protein and Src substrate Tks5/Fish is required for podosome formation and function in Src-transformed fibroblasts. Furthermore, Tks5/Fish is overexpressed in some invasive human cancer cells, and is required for proteolytic invasion. These findings support a broader investigation of Tks5/Fish and its binding proteins both as markers of invasive disease and as potential therapeutic targets. Finally, we have developed a method to form podosomes in cells that do not normally have them, which will allow us in the future to dissect the signals and proteins that regulate podosome formation and turnover.

Experimental procedures

Constructs

The mutant form of chicken Src with an activating Y527F substitution in pSGT and the Tks5/Fish constructs have been previously described (Lock et al., 1998; Abram et al., 2003). All plasmids were double purified over cesium chloride gradients.

Cell lines

NIH3T3 and SrcY527F-transformed NIH3T3 (Src3T3) cells have been described before (Lock et al., 1998). The breast carcinoma cell lines MCF-7, Bt549, Hs578t, and MDA-MB-231 were generously provided by Dr. Han-Mo Koo, Van Andel Institute. The C8161.9 melanoma cell line was generously provided by Dr. Cindy Miranti, Van Andel Institute. T47D and RPMI-7951 cells were obtained from the ATCC. All cell lines were maintained in Dubecco's modified Eagle's medium (DMEM; Invitrogen) containing 10% fetal calf serum (HyClone) and antibiotics in 10% CO_2.

Biochemical techniques

Our general procedures for cell lysis, immunoprecipitation, and immunoblotting have been described before, and are available on request.

Tks5/Fish knockdown cell lines

Src-transformed NIH3T3 cells with reduced levels of Tks5/Fish protein were generated by shRNA technology. Briefly, commercially synthesized complementary primers (Integrated DNA Technologies) were designed to express a short hairpin (sh) RNA for the direct targeting and knockdown of murine *Tks5/Fish* mRNA sequences. These primers were annealed and the resulting construct ligated into pSilencer2.1U6hygro (Ambion) at the accessible BamHI/HindIII restriction sites using standard subcloning techniques. Suc-

cessfully engineered plasmids were confirmed by restriction mapping and by DNA sequencing in the presence of 5% DMSO. Two different shRNA-expression constructs were tested based on previous success with the knockdown of Tks5/Fish in cells transiently transfected with siRNA sequences. A control shRNA sequence (C) was also designed because it does not target murine mRNAs (Ambion). The sequences are:

1: GATCCCGAACGAAAGCGGCTGGTGGTTCAAGAGACCACCAGCCGCTTTCGTTCTTTTTTGGAAA

4: GATCCCGCCAGTCCAACATCTCCTTCTTCAAGAGAGAAGGAGATGTTGGACTGGTTTTTTGGAAA

C: GATCCCACTACCGTTGTTATAGGTGTTCAAGAGACACCTATAACAACGGTAGTTTTTTTGGAAA

The complementary primer sequences are not shown; the siRNA sequences are underlined. shRNA-expression plasmids were introduced into Src-transformed NIH3T3 cells using calcium phosphate. Drug-resistant colonies were selected in the presence of 100 μg/ml hygromycin (Invitrogen) supplemented into the culture medium. Drug-resistant cell lines were maintained in 100 μg/ml hygromycin, except during experimentation.

Transient Tks5/Fish knockdown experiments

Breast carcinoma and melanoma cells were grown to 70% confluency under standard growth conditions. Cells were washed into fresh culture medium 1 hr prior to transfections and into Opti-MEM (Invitrogen) 30 min prior to transfections. Two duplex siRNAs (sense strands: GCCAAAGCAAGGACGAGAU [si1] and AAACCAGUGGCGACCUGAA [si2]) directed against human *Tks5/Fish* (Dharmacon) were used either alone or in combination to knockdown Tks/Fish protein. A "Smartpool" of four siRNAs (Dharmacon) was also used to knockdown human Tks/Fish protein (none of these siRNAs have the same sequence as si1 and si2 above). siRNAs were combined with Lipofectamine 2000 (Invitrogen) according to manufacturer's guidelines and added to cells in Opti-MEM at a final concentration of 100 nM. Control transfections consisted of a pool of four nontargeting siRNAs (duplex control, Dharmacon). Transfections were carried out for 3 hr at 37°C, after which the transfection medium was replaced with fresh culture medium. Analysis of Tks5/Fish protein levels, podosome formation, and transfer into invasion assays were initiated 30 hr posttransfection.

Fluorescence microscopy

Cells were grown for a minimum of 48 hr postpassage on glass coverslips. Cells were fixed in 3% formaldehyde (Electron Microscopy Sciences)/PBS for 10 min, made permeable in 0.1% Triton X-100/PBS for 10 min, and processed with various antibody applications and/or phalloidin in 5% donkey serum (Jackson Immuno-Research Laboratories)/PBS. Primary detection reagents include antibodies for Tks5/Fish (Tks5/Fish.2 1736, 1:500), Src (327), β1-integrin (N-20, 1:400; Santa Cruz), FAK (2A7, 1:200; Upstate Biotechnology), and F actin (Alexa Fluor 488 and 594 phalloidin, 1:1000; Molecular Probes). Secondary detection reagents include Alexa Fluor 488 and 594- and Texas Red-conjugated IgGs (1:2000; Molecular Probes). After washing extensively in PBS, coverslips were mounted in 1 mg/ml p-phenylene diamine (Sigma) in 90% glycerol/PBS. Images were captured using an Axioplan 2 fluorescent microscope and were analyzed with Axiovision 3.0 software (Zeiss).

ECM degradation assay

Fluorescein isothiocyanate (FITC)-labeled gelatin-coated coverslips were prepared essentially as described before (Bowden et al., 2001) with some modifications. Briefly, 2% gelatin (300 bloom; Sigma)/2% sucrose/PBS was prelabeled with 0.2 mg/ml FITC (Fluka) in 50 mM sodium borate (Sigma), 40 mM sodium chloride (Sigma) (pH 9.3) before dialysis into PBS. A thin layer of FITC-gelatin was coated onto glass coverslips and immediately crosslinked with ice-cold 0.8% glutaraldehyde (Electron Microscopy Sciences)/PBS for 15 min at 4°C and then for 30 min at room temperature. Coverslips were successively washed in PBS (3 × 5 min), 5 mg/ml sodium borate/PBS (1 × 3 min), PBS (3 × 5 min), and 70% ethanol (1 × 10 min), before quenching for 1 hr with DMEM. Cells were cultured such that by 48 hr the coverslips were nearly confluent. Coverslips were subsequently processed using standard fluorescence microscopy procedures.

Motility and Invasion assays

Matrigel-containing Boyden transwell chambers (BD Biosciences) were prehydrated 6 hr prior to cell application with medium containing 0.1% fetal calf serum (FCS) placed in the insert chambers and with media containing 10% FCS in the lower chambers. Cells to be tested were detached from cultures dishes in trypsin/EDTA (Invitrogen). This reaction was stopped by a 2-fold dilution in 1 mg/ml soybean trypsin inhibitor/PBS (final concentration). Cells were sedimented at 1000 g for 5 min, suspended in 5 ml PBS, resedimented, and finally suspended in 1 ml of the appropriate growth media containing 0.1% FCS. 25,000 cells (counted by automation; Beckman) in 500 μl of media containing 0.1% FCS were placed into each Boyden chamber. The appropriate growth media containing 10% FCS (750 μl) was placed in the lower chamber to facilitate chemotaxis. Invasion assays were run for 24 (for Src-transformed fibroblasts) or 48 hr (for human cancer cell lines). Noninvading cells were removed from the insert chambers using cotton swabs, then cells which passed through the matrigel membrane were stained with 0.09% crystal violet/10% ethanol. After removing excess stain by washing with deionized water, the stain was eluted with 200 μl of elution buffer (1 part acetate buffer [pH 4.5] : 2 parts ethanol : 1 part deionized water). The optical density of 100 μl of the eluted stain was measured at 570 nm (96-well plate reader, Dynex Technologies). All assays were conducted in triplicate. To study the effect of protease inhibitors on cell invasion, 25,000 cells were suspended in 500 μl of appropriate media containing 0.1% FCS and a cocktail of protease inhibitors, and were placed into each Boyden chamber. The inhibitor cocktail consists of 10 mM calpeptin (03-34-0051; Calbiochem), 20 mM GM6001 (364205; Calbiochem), 10 mg/ml aprotinin (616370; Calbiochem), and 10 mg/ml leupeptin (108975; Calbiochem). Appropriate media (750 μl) containing 10% FCS and the same inhibitor cocktail was placed in the lower chamber. Measurement of invasion was done as described above. Motility assays were conducted in Boyden transwell chambers lacking matrigel over a period of 24 hr. Experimental conditions and the processing of chambers were as described above.

Microinjection

Cells were grown to 50%–60% confluency on etched CellLocate glass coverslips (Eppendorf). All injected DNA samples were adjusted to 100 μg/ml, except where indicated in the figure legend. DNA samples were microinjected into the nuclei of attached cells using either custom-pulled micropipette needles (Flaming/Brown micropipette puller, Sutter Instrument) or prepulled Femtotips needles (Eppendorf). Standardized injection parameters were programmed into a Femtojet Micromanipulator 5171 (Brinkmann) according to manufacturer's guidelines and visualized with an Axiovert 200 inverted fluorescent microscope (Zeiss). To assay podosomes, the cells were incubated 24 hr after injection and processed by standard fluorescence microscopy procedures.

Histochemistry

Slides containing thin sections of dewaxed human breast or skin tissue were warmed in Coplin jars containing 10 mM sodium citrate (pH 6.5) for 20 min at 90°C before being allowed to cool back to room temperature. With interspersed washes in PBS, all samples were prepped for antibody application by successive 30 min treatments in 0.1% Triton-X 100/PBS, 3% hydrogen peroxide, and 1% BSA/5% donkey serum/PBS. With interspersed washes in PBS, Tks5/Fish detection was procured using a polyclonal antibody to the first SH3 domain of Tks5/Fish (D7771) at 1:100 dilution in 5% donkey serum/PBS for overnight at 4°C, a biotinylated anti-rabbit secondary antibody (Santa Cruz) at 1:100 dilution in 5% donkey serum/PBS for 1 hr at room temperature, and an HRP-conjugated anti-biotin tertiary antibody (Vectastain, Vector Laboratories) used according to manufacturer guidelines. Slides were developed using 3,3′ diaminobenzidine as substrate (DAB Substrate Kit, Vector Laboratories) according to manufacturer's guidelines. Alternatively, slides were stained with hematoxylin and eosin. Images were obtained using an Eclipse E600 bright field microscope (Nikon) and were digitally captured using Spot Advanced 4.0.4 software (Diagnostics Instruments).

Zymography

100,000 cells (counted by automation; Beckman) in 2 ml of DMEM containing 10% FCS were placed in single wells of a 6-well dish and grown to 80% confluency. Cells were washed 2 times in 2 ml PBS and then incubated with 2 ml Opti-MEM for 48 hr. Conditioned medium was collected from the cells, filtered at 0.2 μm, and diluted with zymogram sample buffer (Bio-Rad). Samples were analyzed on 10% acrylamide/gelatin Criterion gels

(Bio-Rad). Gels were processed by stepwise treatments in renaturation buffer for 1 hr, development buffer for overnight, Coomassie Brilliant Blue R-250 staining solution for 1 hr, and destaining in 10% methanol/10% acetic acid (Bio-Rad). The 72 kDa band is MMP2 (gelatinase A) and the 95 kDa band is MMP9 (gelatinase B).

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