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Adhesion signaling by a novel mitotic substrate of src kinases

Ami S Bhatt[1], Hediye Erdjument-Bromage[2], Paul Tempst[2], Charles S Craik[1,3] and Mark M Moasser*[,4]

[1]*School of Medicine, University of California, San Francisco, CA 94143, USA;* [2]*Program in Molecular Biology, Memorial Sloan-Kettering Cancer Center, Sloan-Kettering Institute, New York, NY 10021, USA;* [3]*Department of Pharmaceutical Chemistry, University of California, San Francisco, CA 94143, USA;* [4]*Department of Medicine, University of California, San Francisco, CA 94143, USA*

Src kinases are activated and relocalize to the cytoplasm during mitosis, but their mitotic function has remained elusive. We describe here a novel mitotic substrate of src kinases. Trask (*tr*ansmembrane and *a*ssociated with *s*rc *k*inases) is a 140 kDa type I transmembrane glycoprotein unrelated to currently known protein families. Src kinases phosphorylate Trask *in vitro* and mediate its mitotic hyperphosphorylation *in vivo*. Trask associates with both yes and src, is localized to the cell membrane during interphase, and undergoes cytoplasmic relocalization during mitosis. Overexpression of Trask leads to cell rounding and a loss of adhesion phenotype. Consistent with a function in cell adhesion, Trask interacts with a number of adhesion and matrix proteins including cadherins, syndecans, and the membrane-type serine protease 1 (MT-SP1), and is proteolytically cleaved by MT-SP1. Trask is unique among cell adhesion molecules in that it is under cell cycle regulation and thus links src kinases with the mitotic regulation of cell adhesion. This suggests a potential pathway by which hyperactive src kinases in tumors can deregulate adhesion signaling and mediate the metastatic phenotype.
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Introduction

Tyrosine kinases are important regulators of growth in multicellular organisms. Deregulation of tyrosine kinase signaling leads to abnormal growth and development, cancer, and metastasis. A fundamental challenge of molecular oncology is to understand the specific cellular and extracellular functions of tyrosine kinase families in order to understand their oncogenic functions. A potent oncogenic potential is inherent within members of the src family of nonreceptor tyrosine kinases. When constitutively activated in experimental systems, src kinases are transforming in tissue culture and transgenic models (Jove and Hanafusa, 1987; Guy *et al*., 1994; Webster *et al*., 1995). The viral oncogenes v-*src*, v-*yes*, and v-*fgr* are activated homologs of cellular src proteins and are responsible for producing tumors in animal hosts. High src activity is characteristic of many common human tumors including colon cancers, breast cancers, lung cancers, sarcomas, and melanomas (Rosen *et al*., 1986; Cartwright *et al*., 1989; Ottenhoff-Kalff *et al*., 1992; Irby and Yeatman, 2000). In a subset of these tumors, this is due to mutational activation of src (Mao *et al*., 1997; Irby and Fujita, 1999; Sugimura *et al*., 2000). The interest in determining a src signaling pathway has led to the identification of numerous src substrates involved in diverse cellular functions including growth factor receptor signaling, cytoskeletal organization, cell motility, and adhesion signaling (Erpel and Courtneidge, 1995). Src function is also required for cell cycle progression, as microinjection of anti-src/yes/fyn antibodies prevents G1/S transition and microinjection during G2 prevents G2/M progression (Roche *et al*., 1995a, b). The G1/S activities of src are in part regulated through inhibition by RACK1 (Mamidipudi *et al*., 2004). Although the activities of src kinases are required for cell cycle progression in mammalian cells, the cycle-specific cellular functions that they perform have remained elusive.

Much indirect and direct evidence suggests that src is important in the mitotic phase of the cell cycle. Src is predominantly associated with the plasma membrane, however during G2/M is redistributed to the cytoplasmic compartment (David-Pfeuty and Nouvian-Dooghe, 1990). Src kinase activity is increased during mitosis in association with amino-terminal serine and threonine phosphorylations as well as partial dephosphorylation of tyrosine 527 by RPTPalpha (Chackalaparampil and Shalloway, 1988; Bagrodia *et al*., 1991; Park and Cartwright, 1995; Mustelin and Hunter, 2002). Dephosphorylation of tyrosine 527 leads to the release of intramolecular interactions and increased accessibility of

*Correspondence: MM Moasser, Department of Medicine, University of California, San Francisco, UCSF Box 0875, 2340 Sutter St, Rm 144, San Francisco, CA 94143, USA;
E-mail: mmoasser@medicine.ucsf.edu
The Trask sequence has been deposited in the NCBI database with Genebank accession #AY167484
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the SH2 and SH3 domains for interaction with proteins specifically during mitosis (Bagrodia *et al*., 1994). Only one mitotic substrate of src, Sam68, has been identified so far (Fumagalli *et al*., 1994; Taylor *et al*., 1995). Sam68 is an RNA binding protein and its RNA binding activity is abolished when tyrosine phosphorylated by src kinases (Wang *et al*., 1995). The role of Sam68 phosphorylation with regard to the cell cycle and the RNA targets of Sam68 that mediate mitotic events remain to be defined (Lukong and Richard, 2003).

Considerable evidence suggests that the transforming functions of src are mediated through alterations in cell migration and invasiveness rather than increased proliferative rate. Src-transformed fibroblasts have a much higher metastatic potential compared to their ras-transformed counterparts (Tatsuka *et al*., 1996). In other experimental xenograft models, src kinase activity is not required for tumorigenic growth in mice, but is essential for the development of metastases (Boyer *et al*., 2002). Consistent with a prometastatic function, elevated src activity is associated with alterations in cell adhesion, not increased proliferative rate (Jones *et al*., 2002).

Based on a hypothesis that cell cycle substrates of src kinases may be highly relevant to the cancer phenotype, we have used a human breast cancer model with activated src kinases to search for cell cycle phase-specific substrates of the src family members src and yes, and report here the purification and identification of a novel mitotic substrate of src kinases. Experimental overexpression of this transmembrane glycoprotein reveals a function in cell adhesion and suggests that src kinases may signal the characteristic cell adhesion changes of epithelial cells undergoing mitosis.

Results

Purification of p85

MDA-468 breast cancer cells have activated src and yes kinases (Moasser *et al*., 1999). Immunoprecipitation of src and yes from these cells during each of the phases of the cell cycle revealed the co-immunoprecipitation of an 85 kDa tyrosine-phosphorylated protein predominantly within yes immune complexes (Figure 1a). This protein is hyperphosphorylated specifically in mitotic cells blocked with nocodazole but not in mitotic cells blocked with an src-selective tyrosine kinase inhibitor, leading us to suspect that it may be a mitotic substrate of src kinases (Figure 1, lanes 5 and 6). Immunoblot analyses failed to determine the identity of this 85 kDa protein; therefore, we sought to purify it. Purification was performed using anti-phosphotyrosine antibodies immobilized on solid support to purify p85 from mitotic lysates of MDA-468 cells. The purified p85 band was isolated by SDS–PAGE and subjected to trypsin digestion, and two selected tryptic peptides were sequenced. The two sequences showed no homology with described proteins or cDNAs; however, they matched with the same potential open reading frame (ORF) of a human Est. The corresponding IMAGE clone contained a 1 kb partial cDNA with no identity within the cDNA and protein databases including NCBI, EMBL, or DDBJ (Japan). Northern blots probed with the radiolabeled partial cDNA revealed that p85 is encoded by a 6 kb RNA message with increased expression in mitotic cells (shown in Figure 2b). The full-length p85 cDNA sequence was then determined using 5′ and 3′ RACE PCR and this sequence information was then used to design primers and subsequently clone a 5 kb cDNA by RT–PCR using RNA from mitotic MDA-468 cells.

p85 encodes a novel transmembrane protein

The ORF of the p85 cDNA was determined from the purified peptide sequence information. A number of potential ATG start codons lie within the upstream sequences of this ORF of which the ATGs at positions 93, 669, and 651, in decreasing order, comply best with Kozak consensus translational start sites (Kozak, 1987). These regions identify coding regions of 2508, 1950, and 1932 bp within the same ORF encoding proteins of 835, 649, and 643 aa with predicted molecular weights of 91, 73, and 72 kDa. This ORF is followed by a 3.5 kb 3′ untranslated region. No other significant ORFs are found within the 6 kb p85 cDNA.

Comparison of the 835 aa p85 sequences against protein or cDNA databases does not reveal close homology with any currently known families of proteins. There are no homologs in procaryotic or primitive eucaryotic organisms, and close homologs of this protein are only found in more complex organisms. A transmembrane region is identified at amino-acid position 665–686 with high probability using all major algorithms (PSORT, DAS, SMART, SOSUI, TMPRED) with an orientation that predicts a large extracellular N-terminal region and a smaller intracellular C-terminal region (Figure 1b). Consistent with a predicted extracellular localization, there are consensus signal peptide sequences at position 1–29 of the 835 aa protein. Optimal Kozak translational sequences and 5′ sequences encoding a conserved signal peptide motif identify that the ATG start site at position 93 marks the correct translation initiation site and p85 is an 835 aa protein encoded by a 2508 bp coding region. Amino-acid positions 223–348 and 416–544 are identified with low scores as CUB domains (only one of the two is identified by SMART and the other identified by Pfam). There are five tyrosine residues within the intracellular domain (ICD) and a proline-rich region at 766–799 (identified by BLOCKS). This protein was named Trask (*tr*ansmembrane and *a*ssociated with *s*rc *k*inases) and is referred to as such from this point onwards.

Trask sequences were used to design peptide immunogens and two monoclonal antibodies were successfully raised against Trask sequences (clones 12F3 and 2G4). On immunoblot analyses of MDA-468 lysates, these antibodies predominantly bind an 85 kDa protein that appears as a doublet consistent with phosphory-

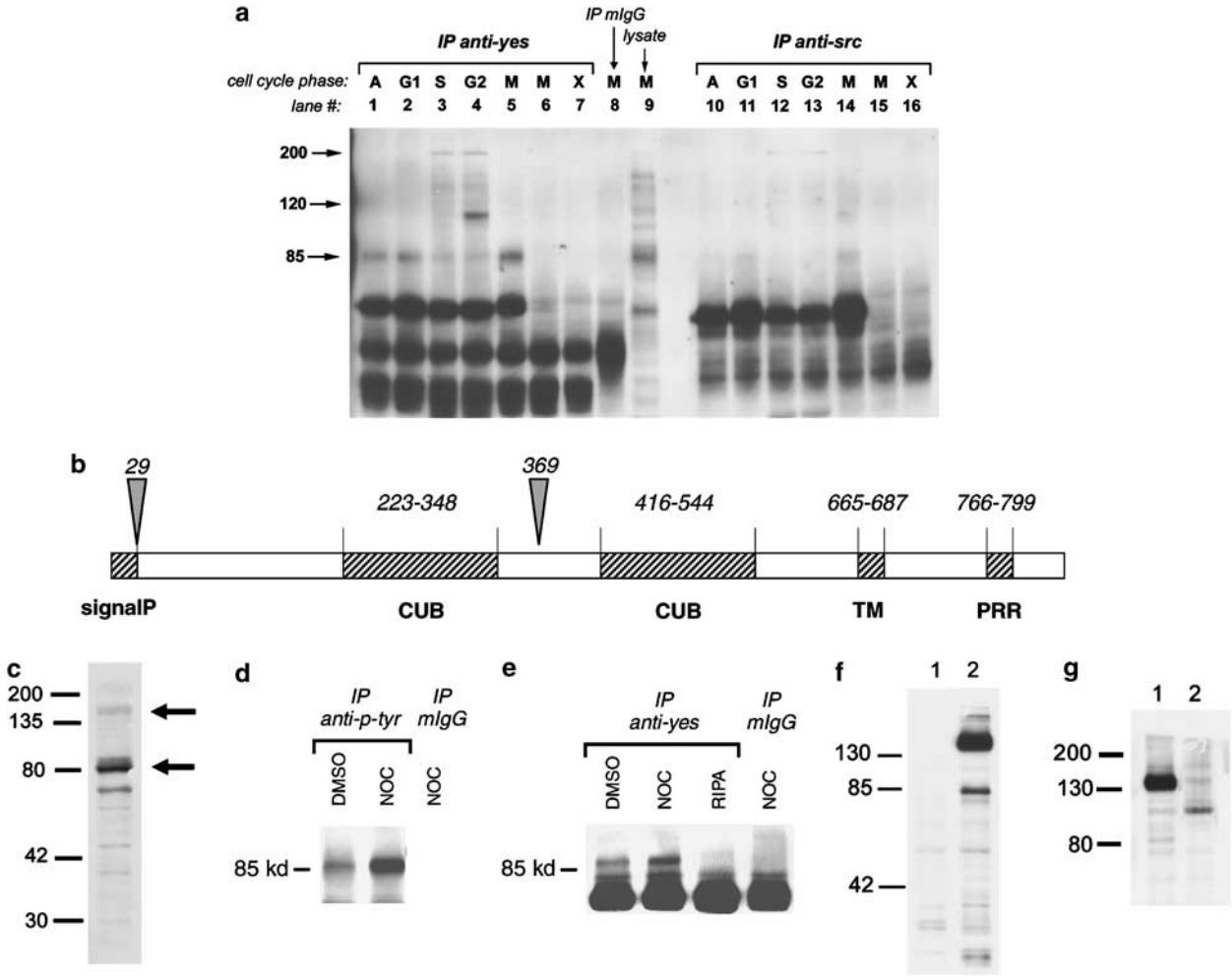

Figure 1 Identification and characterization of an 85 kDa mitotic phosphoprotein associated with yes. (**a**) An 85 kDa phosphoprotein was identified by anti-phosphotyrosine immunoblotting of anti-yes and anti-src immune complexes. Lanes correspond to asynchronous cells (1 and 10) or cells synchronized in G1 (2 and 11), S (3 and 12), G2 (4 and 13), or M (5 and 14) phases of the cell cycle using lovastatin, aphidicolin, etoposide, and nocodazole, respectively. Cells in lanes 6 and 15 were blocked in mitosis by the src-selective tyrosine kinase inhibitor PD173955. Additional lanes include immunoprecipitates lacking cell lysates (7 and 16), or mitotic mIgG immunoprecipitates (8), or total lysate (9). (**b**) Structural organization of Trask protein. Labels indicate the signal peptide (SignalP), two CUB domains, transmembrane domain (TM), and proline-rich region (PRR). Arrows indicate two known cleavage sites described in the text. (**c**) Immunoblot of MDA-468 cells using anti-Trask 12F3 antibodies. (**d**) Anti-phosphotyrosine immunoprecipitates from asynchronous and mitotic MDA-468 cells were immunoblotted with anti-Trask antibodies. (**e**) Anti-yes immunoprecipitates from MDA-468 cells were immunoblotted with anti-Trask antibodies. Controls include immunoprecipitates from lysis buffer without cell lysate (lane 3) and mIgG control (lane 4). (**f**) MCF-7 cells were transfected with vector control (1) or pcDNA4-MycHis-Trask (2). An anti-myc immunoblot is shown here. Similar results were obtained by transfecting 293T or MDA-468 cells (not shown). (**g**) MDA-468 cells were transiently transfected with pcDNA4-MycHis-Trask and treated with DMSO (1) or tunicamycin (2) for 24 h. An anti-myc immunoblot is shown here

lated and unphosphorylated forms (Figure 1c). Immunoprecipitation followed by immunoblot analyses confirm that Trask is associated with yes and to a smaller degree with src and is markedly tyrosine phosphorylated during the mitotic phase in MDA-468 cells (Figure 1d and e). These studies confirm that the purified protein is indeed the mitotic phosphoprotein associated with yes and not a background copurification product.

Transient transfection of a construct containing the full 2508 bp ORF with a C-terminal myc tag (pcDNA4-MycHis-Trask) reveals that Trask is actually expressed as a 140 kDa protein and a secondary 85 kDa product (Figure 1f). A faint 140 kDa band is also seen in the endogenous Trask immunoblots of MDA-468 cells although it is not the predominant form of Trask in these cells (Figure 1c). The 85 kDa form of Trask is a product of proteolytic cleavage and is described further below. Treatment of MDA-468 cells with the glycosylation inhibitor tunicamycin decreases the molecular weight of myc-tagged Trask from 140 to 100 kDa, revealing that glycosylation accounts for a substantial increase in molecular weight above that predicted from its primary amino-acid sequence (Figure 1g).

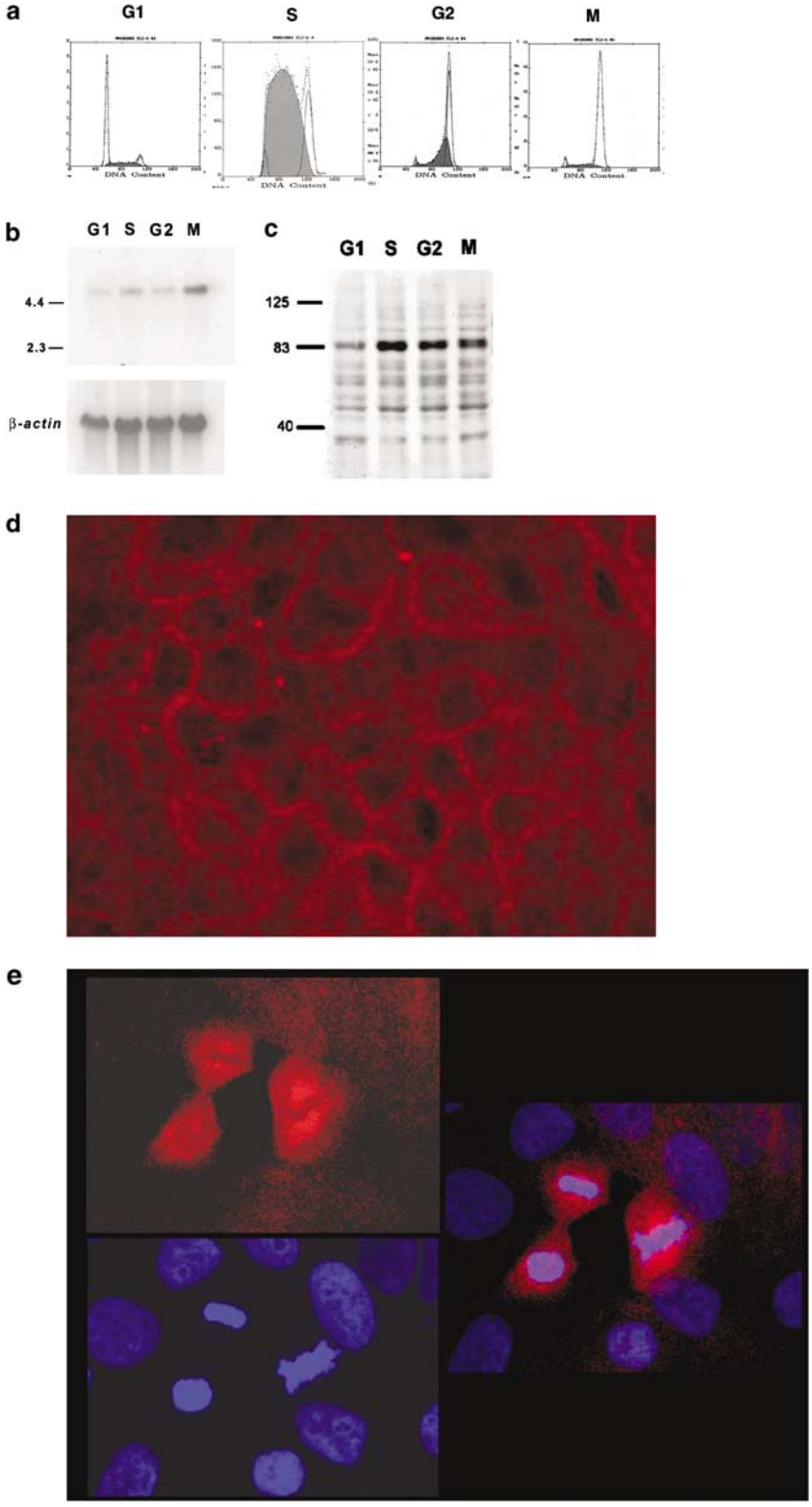

Figure 2 Cellular expression of Trask. (**a**) MDA-468 cells were synchronized in G1, S, G2, or M and cell cycle phase was verified. (**b**) Northern blot analysis of polyA-selected RNA from these cells was performed using a 2 kb ^{32}P-labeled Trask cDNA probe. (**c**) Immunoblot analysis of total lysates from these cells was performed using anti-Trask 12F3 monoclonal antibodies. (**d**) Immunofluorescence microscopy of MCF10A breast epithelial cells using anti-Trask monoclonal antibodies labeled with rhodamine red. The majority of the cells show cell membrane staining as shown in this image. Mitotic cells were identified by their characteristic chromatin condensation and show cytoplasmic staining. A representative high-power field is shown in image (**e**)

Cellular expression of Trask

Northern blot analysis using a 2 kb coding region Trask cDNA probe shows low expression during most of interphase with a slight increase during S phase, but a fourfold induction of expression during the mitotic phase (Figure 2a and b). Aliquots of the same cells were lysed and studied by Western blotting using anti-Trask 12F3 antibodies. This analysis shows a high level of Trask protein expression during the S, G2, and M phases compared to G1 (Figure 2a and c). The localization of Trask was studied by immunofluorescence microscopy using anti-Trask antibodies. Confocal microscopic analyses of a number of cell types including MCF10A, MDA-468, Du-145, and HT29 cells show punctate and predominantly membrane staining as predicted by the encoded transmembrane domain (Figure 2d). This localization was seen using either 12F3 or 2G4 antibodies and using either paraformaldehyde or methanol fixation methods. Mitotic cells were identified within the asynchronous population by their characteristic chromatin condensation. Unlike interphase cells, many mitotic cells show predominantly cytoplasmic immunolocalization of Trask (Figure 2e). The punctate staining pattern and the cytoplasmic redistribution of Trask may reflect localization to the endosomal membrane, as observed with src (Kaplan *et al*., 1992).

Src kinases phosphorylate Trask

In order to determine whether Trask is a substrate of src kinases, recombinant Trask protein was synthesized. A 460 bp fragment encoding the entire ICD of Trask fused with carboxy-terminal V5 and His tags was expressed in *Escherichia coli* DH5α cells, purified over a charged nickel affinity column, and subsequently dialysed against PBS. Gel electrophoresis and immunoblot analyses of the purification products using anti-V5 antibodies confirm expression of a 30 kDa recombinant fusion protein with a purity of approximately 80% by Coomassie blue staining (Figure 3a). *In vitro* kinase reactions were performed with recombinant src and with immunopurified yes proteins using the purified recombinant Trask ICD as the substrate and ^{32}P-labeled ATP. In this reaction, both src and yes phosphorylate the Trask ICD (Figure 3b and c).

To determine whether src kinases also phosphorylate Trask *in vivo*, we treated MDA-468 cells with the tyrosine kinase inhibitor PD173955, which is selective for members of the src family. PD173955 is equally active against both src and yes *in vitro* (Moasser *et al*., 1999). Trask is dephosphorylated within 5 min of exposure to PD173955, suggesting that it is a substrate of src kinases *in vivo* (Figure 3d). Although Trask is tyrosine phosphorylated in interphase in MDA-468 cells, it undergoes significant hyperphosphorylation

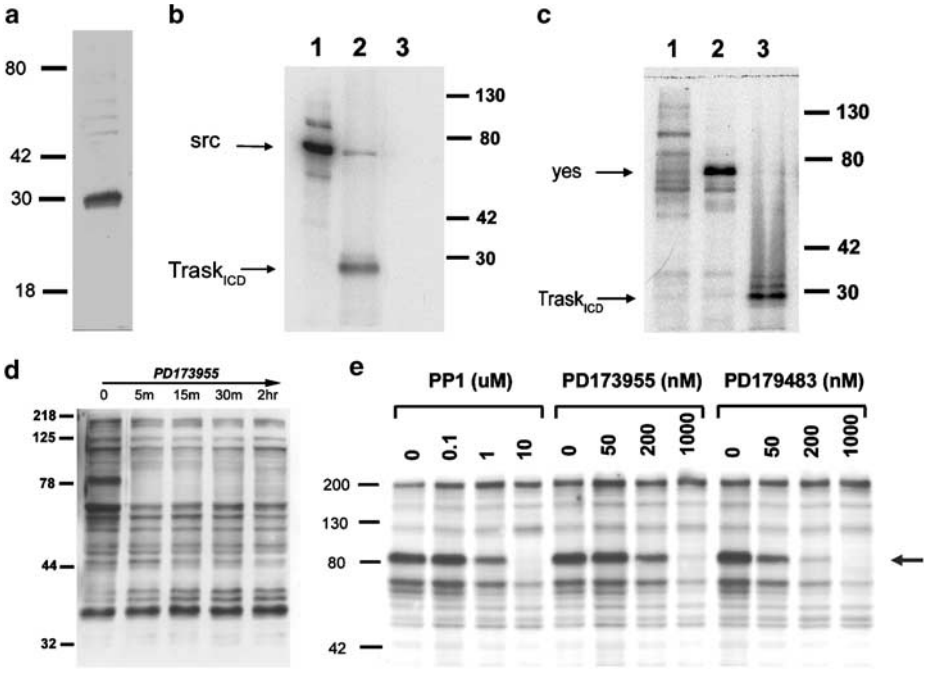

Figure 3 Src kinases phosphorylate Trask *in vitro* and *in vivo*. (**a**) The purity of recombinant Trask$_{ICD}$ was verified by SDS–PAGE separation and Coomassie blue staining. (**b**) *In vitro* src kinase reaction. Lanes 1–3 correspond to reactions containing src alone, src and Trask$_{ICD}$, and Trask$_{ICD}$ alone. (**c**) *In vitro* yes kinase reaction. Lanes 1–3 correspond to reactions containing mIgG immunoprecipitates and Trask$_{ICD}$, α-yes immunoprecipitates alone, and α-yes immunoprecipitates and Trask$_{ICD}$. (**d**) Asynchronous MDA-468 cells were treated with 1 μM P173955 for the indicated times and lysates were immunoblotted using anti-phosphotyrosine antibodies. The 85 kDa phosphotyrosine band indicated by the arrow is Trask, identified previously by purification. (**e**) MDA-468 cells were treated for 20 h with nocodazole, and mitotic shake-offs were washed and released into media containing the indicated concentrations of src-selective tyrosine kinase inhibitors for 30 min. Negative control lanes were treated with vehicle (DMSO) and are labeled as '0'. An anti-phosphotyrosine immunoblot is shown here. The 85 kDa band identified by the arrow is phosphorylated Trask, and disappears rapidly with increasing concentrations of src inhibitors

during mitosis. To determine whether src kinases also mediate this mitotic hyperphosphorylation, we released nocodazole-blocked MDA-468 cells for short times into media containing three different tyrosine kinase inhibitors selective for the src family (Kraker *et al*., 2000; Mizenina and Moasser, 2004). Each of these compounds inhibits the mitotic hyperphosphorylation of Trask in a dose-dependent manner (Figure 3e). Although each of these tyrosine kinase inhibitors may have some non-specific activities and inhibit cellular kinases other than src and yes, all three inhibitors give similar results. These data suggest that the *in vivo* mitotic tyrosine phosphorylation of Trask is mediated through the src family kinases, of which src and yes are expressed in these cells.

Trask is an adhesion protein

In order to study the cellular functions of Trask, we established an inducible expression system to overexpress it in MDA-468 cells. MDA-468 cells were first stably transfected with the pcDNA6/TR vector to express the tet-repressor protein and subsequently transfected with a pcDNA4-TO-MycHis vector containing the full-length Trask cDNA in-frame with C-terminal myc and His tags. Induction of these transfectants (MDA-468TR-Trask) with doxycycline leads to overexpression of p140Trask as well as p85-Trask, again confirming that the two forms are products of the same transcript (Figure 4a). Overexpression of

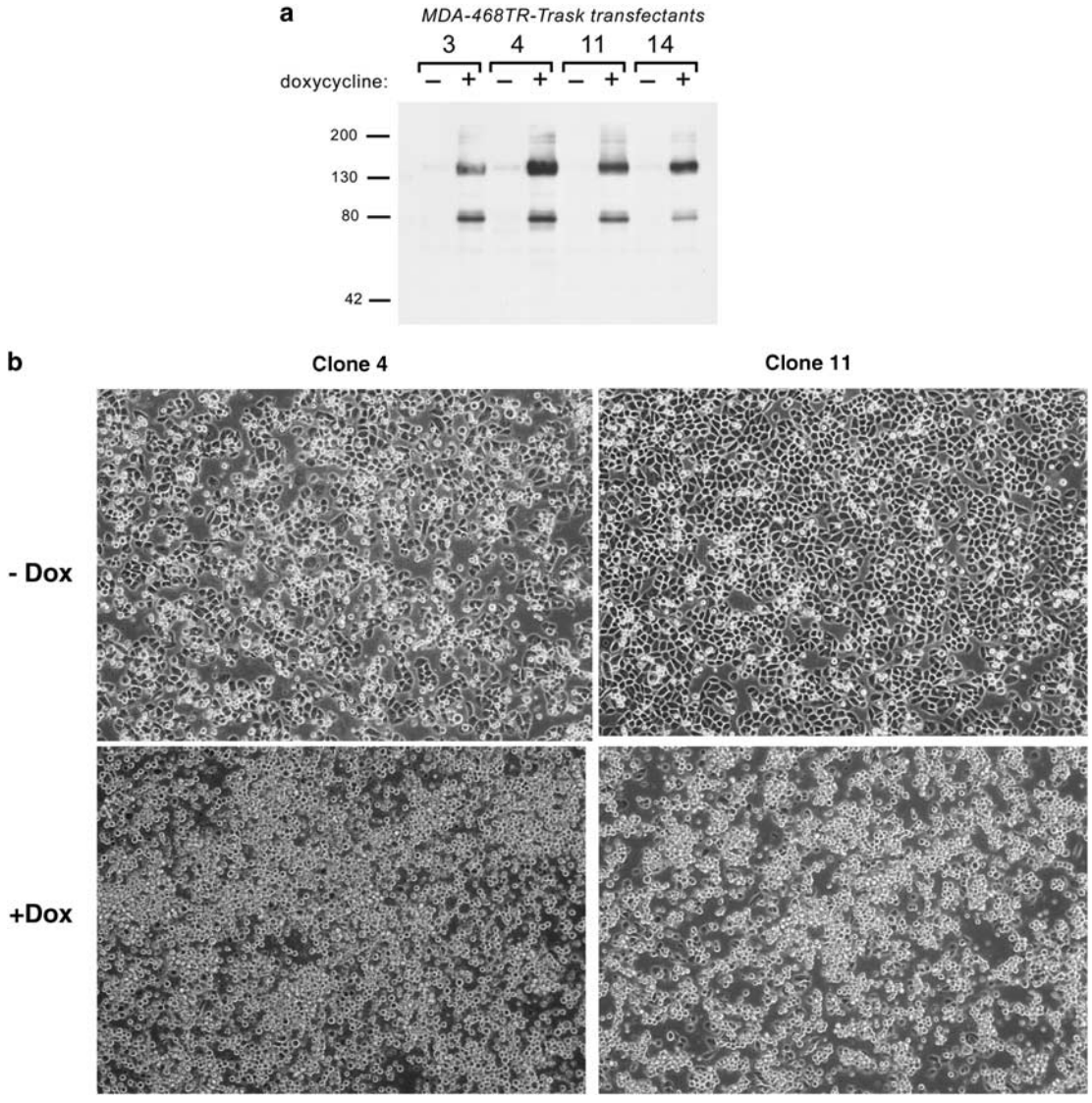

Figure 4 Trask overexpression in MDA-468 cells. (**a**) MDA-468TR cells were transfected with pcDNA4-TO-MycHis-Trask and stable transfectants (named MDA-468TR-Trask) selected in Zeocin. Inducible expression of the myc-tagged Trask protein is shown here by anti-myc immunoblots of four clones. Induction of expression of full-length Trask cDNA results in expression of a 140 kDa full-length Trask protein as well as an 85 kDa cleaved product. (**b**) MDA-468TR-Trask cells were grown in the absence or presence of 100 ng/ml doxycycline and inspected under inverted light microscopy. Induction with doxycycline results in cell rounding and continued growth in a loosely adherent semisuspended state morphology. Clones 4 and 11 are shown here and are typical of all transfected clones. Doxycycline treatment of vector-transfected controls has no visible effects on cell morphology (not shown)

Trask has no effect on the proliferative rate of MDA-468 cells measured in assays of monolayer growth or anchorage-independent growth (data not shown). However, overexpression of Trask has a profound effect on cell shape and adhesion, easily apparent under light microscopy. When induced to overexpress Trask, MDA-468 cells develop a rounded shape, lose their flattened adherent morphology, and grow in a loosely adherent suspension (Figure 4b). This effect is seen in all transfectants that overexpress Trask and not in doxycycline-treated vector controls (not shown).

Since the transfection data suggest a function in cell adhesion, we explored Trask interactions with other proteins involved in cell adhesion and cell matrix association. MDA-468TR-Trask cells were induced to overexpress Trask and a number of candidate membrane, matrix, and adhesion proteins were immunoprecipitated with specific antibodies. We looked for the co-immunoprecipitation of Trask in these immune complexes by anti-myc immunoblotting. These studies identify that Trask interacts with the adhesion proteins N-cadherin and P-cadherin, the matrix proteins syndecans 1 and 4, and the membrane serine protease MT-SP1 (Figure 5a). The interaction between Trask and MT-SP1 may be mediated by the CUB domains in both proteins as CUB domains are thought to play a role in protein–protein interactions in the extracellular environment. These studies were also repeated by anti-myc immunoprecipitations, which further confirm the interactions with N-cadherin, P-cadherin, and MT-SP1 (Figure 5b) Thus far we have not been able to detect an interaction between Trask and selected members of the integrin family.

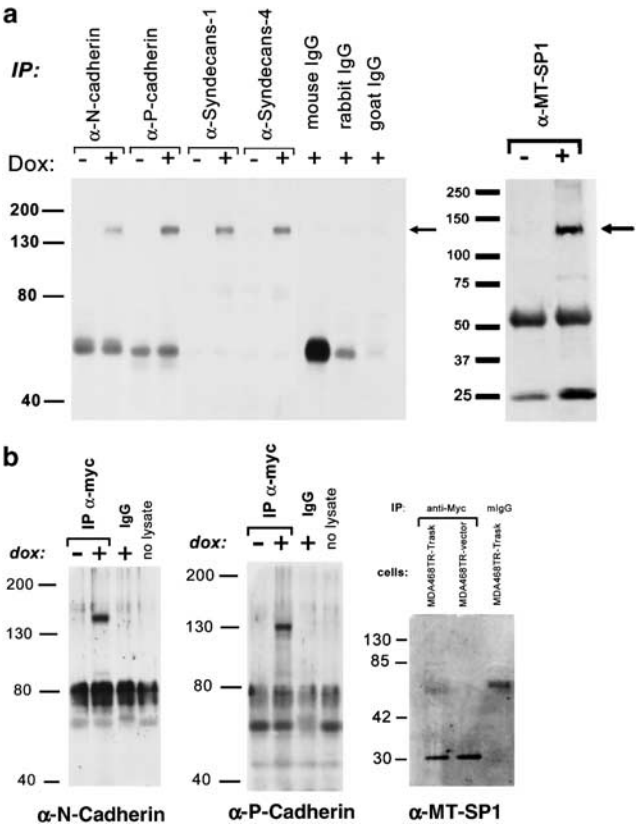

Figure 5 Trask interactions with membrane and matrix proteins. (**a**) MDA-468TR-Trask cells were induced with doxycycline to express Trask, and cell lysates were immunoprecipitated with the indicated specific antibodies. Immune complexes were separated by SDS–PAGE and immunoblotted using anti-myc antibodies to detect the presence of myc-tagged Trask in the complexes. Controls include immune complexes using nonimmune IgGs and immune complexes from uninduced cells that do not express the construct. (**b**) The same experiments were performed in reverse order such that lysates were immunoprecipitated with anti-myc antibodies and immunoblotted with the indicated specific antibodies

Trask is a serine protease substrate

The expression of 140 and 85 kDa forms of Trask from the same cDNA raises the possibility that p85Trask may be a cleavage product of p140Trask. Its interaction with the membrane serine protease MT-SP1 further strengthens this hypothesis. The induction of Trask expression in MDA-468TR-Trask cells in the presence of broad serine protease inhibitors results in a reduction in the amount of the 85 kDa product. More specifically, the trypsin-fold-specific macromolecular serine protease inhibitor ecotin abrogates cleavage of p140, suggesting that an extracellular trypsin-fold serine protease induces Trask processing (Figure 6a). To determine whether MT-SP1 cleaves Trask, we generated recombinant extracellular domain of Trask ($rTrask_{ECD}$) to test as a substrate for MT-SP1 *in vitro*. The entire Trask extracellular domain (ECD) was cloned into pcDNA4-TO-MycHis in-frame with c-terminal Myc and His tags and transfected into 293T cells. Due to the presence of signal peptide sequences and the absence of a transmembrane domain, the expressed product is secreted into the conditioned media (Figure 6b). 293T-$Trask_{ECD}$ cells were grown in serum-free media and $rTrask_{ECD}$ was collected from the conditioned media, purified over a charged nickel column, and dialysed against PBS (Figure 6c). $rTrask_{ECD}$ was incubated with recombinant catalytic domain of MT-SP1 ($rMT\text{-}SP1_{CD}$) *in vitro* and the reaction products were separated and identified by anti-myc immunoblots and silver staining (Figure 6d and e). In this reaction, $rMT\text{-}SP1_{CD}$ completely cleaves $rTrask_{ECD}$ into two fragments. Our $rTrask_{ECD}$ preparation prepared from 293T cells contains some amount of cleaved Trask as seen in both the silver stain and the myc immunoblots; however, addition of $rMT\text{-}SP1_{CD}$ cleaves it completely. N-terminal amino-acid sequencing of the reaction products revealed a free N-terminal sequence of KFVPGCF, thereby identifying that MT-SP1 cleaves Trask after Arg^{369}. MT-SP1 is well established as a trypsin specificity protease cleaving after the basic amino acids arginine and lysine. Using a P1-fixed combinatorial peptide substrate library approach, we previously reported the extended MT-SP1 cleavage site preferences to be K/R in P4, R/K/Q in P3, and S in P2 (Takeuchi *et al*., 2000). The sequence KQS at P4, P3, and P2 of the Trask cleavage site matches precisely with these MT-SP1 substrate preferences. N-terminal sequencing of the N-terminal

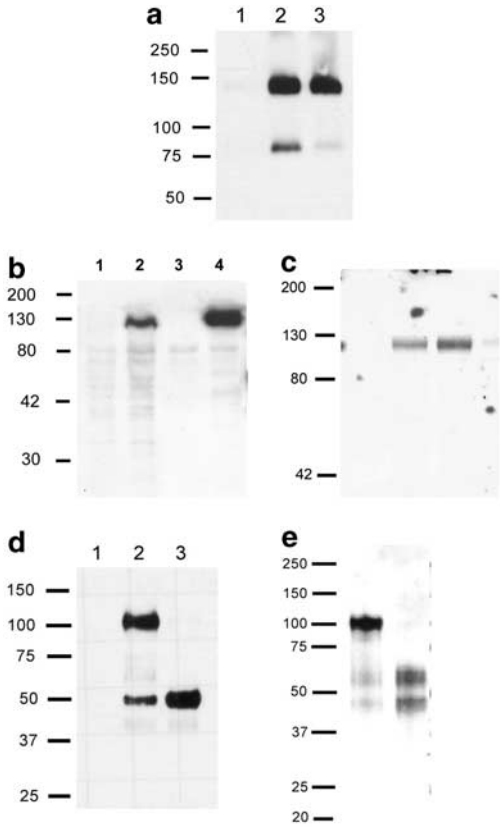

Figure 6 Trask cleavage by MT-SP1. (**a**) MDA-468TR-Trask cells were left uninduced (lane 1) or induced to express Trask with 100 ng/ml doxycycline (2 and 3). The induced cells were treated with no drug (2), or with the serine protease inhibitor ecotin (7 μM) (3) beginning 2 h before doxycycline induction. At 24 h after doxycycline induction, cell lysates were harvested and immunoblotted using anti-myc antibodies. The expression of p85Trask is reduced by the protease inhibitor. (**b**) 293T cells were transfected with a myc/his-tagged Trask $_{ECD}$ construct. Anti-myc immunoblots were performed to confirm the expression of $Trask_{ECD}$ in cell lysates from controls (1) and transfectants (2), and the secretion of $Trask_{ECD}$ in the media of controls (3) and transfectants (4). (**c**) These transfected 293T cells were grown in serum-free media and the soluble recombinant $Trask_{ECD}$ was purified from conditioned media over a charged nickel column and dialysed against PBS yielding a pure 120 kDa soluble TraskECD product shown here by Coomassie staining. (**d**) A 1 μg portion of $rTrask_{ECD}$ was added to an *in vitro* protease reaction using recombinant catalytic domain of MT-SP1 ($rMT\text{-}SP1_{CD}$). The mixture was incubated for 1 h at 37°C in PBS, and the reaction products were denatured, deglycosylated by PNGaseF treatment, separated on SDS—PAGE, and immunoblotted using anti-myc antibodies. Lanes correspond to reactions containing $rMT\text{-}SP1_{CD}$ alone (1), $rTrask_{ECD}$ alone (2), or $rMT\text{-}SP1_{CD}$ and $rTrask_{ECD}$ (3). The $rTrask_{ECD}$ construct is myc-tagged at the carboxy-terminus; therefore, these anti-myc immunoblots identify uncleaved ECD as well as the carboxy-terminal cleavage product, but not the N-terminal cleavage product. (**e**) Silver staining of $rTrask_{ECD}$ before (1) and after (2) cleavage with $rMT\text{-}SP1_{CD}$ identifies both cleavage products. Our $rTrask_{ECD}$ preparation from 293T cells contains some amount of cleaved Trask as seen in both the silver stain and the myc immunoblots here; however, addition of $rMT\text{-}SP1_{CD}$ results in complete proteolysis. The precise site of the *in vitro* cleavage was determined by N-terminal sequencing of the reaction product, as described in the text

fragment of Trask after processing was FEIAL, consistent with cleavage of the signal peptide after Ala[29].

Discussion

A potential mitotic function of src proteins has been the subject of speculation for more than a decade. However, direct analysis of this potential cell cycle function has been awaiting the identification of a mitotic substrate of src kinases. The first such substrate was Sam68, an RNA binding protein whose RNA binding activity is inhibited by tyrosine phosphorylation (Fumagalli *et al*., 1994; Taylor and Shalloway, 1994). The role of this phosphorylation in regulating mitotic events remains to be defined. We present here a second mitotic substrate of src kinases, a transmembrane protein that functions in cell adhesion. The cell cycle and adhesion functions of this novel src substrate suggest the new hypothesis that src may regulate the mitotic changes in cell adhesion. Cell adhesion is generally presumed to be regulated during mitosis, particularly in epithelial cells, which need to release themselves from their cell–matrix and cell–cell interactions transiently in order to allow cell duplication and separation of daughter cells. However, the molecular mechanism that regulates this dynamic cell adhesion process is unknown. Using an inducible overexpression model, we show that Trask functions in cell adhesion. Trask is unique among currently described cell adhesion molecules in that it is under cell cycle regulation and is a candidate protein for regulating cell adhesion during mitosis.

The adhesion functions of Trask could be regulated through its phosphorylation. In our inducible Trask overexpression model, the induced Trask, unlike endogenous Trask, undergoes significant interphase tyrosine phosphorylation while producing a loss of adhesion phenotype. The excessive interphase phosphorylation of the induced construct may be due to the excessive nature of its expression in this model system. Whether the phosphorylation of Trask is the cause or the consequence of the loss of adhesion remains to be determined. Phosphorylation of Trask could mediate structural changes in its conformation leading to altered interaction of its ECD, or could promote or inhibit its interaction with other transmembrane proteins involved in cell adhesion. Phosphorylation could also recruit cytoplasmic signaling proteins, in particular SH2-containing proteins involved in adhesion signaling. These are potential mechanisms by which Trask phosphorylation could influence interactions with other cell adhesion proteins and signal changes in cell adhesion.

The mitotic phosphorylation of Trask also potentially implicates src kinases in the regulation of Trask function and ultimately in the mitotic regulation of cell adhesion. In addition to MDA-468 cells, the mitotic hyperphosphorylation of Trask is evident in a number of other epithelial tumor cell lines with activated src kinases, but not readily apparent in an immortalized breast epithelial cell line or in fibroblasts (not shown).

In addition to its phosphorylation, the expression and localization of Trask are also regulated during the cell cycle. While the Trask RNA remains low during most of interphase with a small increase during S phase, Trask protein expression increases during S, G2, and M phases. This is likely due to changes in mRNA stability and other post-transcriptional or post-translational mechanisms of regulation. Taken together, there is clear evidence of the cell cycle regulation of Trask at the level of expression, localization, and post-translational modification by phosphorylation.

Trask function and expression may be important in human tumor progression. Consistent with this, Trask has also been identified by two other groups using global strategies to identify tumor-associated genes. Using cDNA chip hybridization techniques to search for genes preferentially expressed in solid tumors relative to normal tissues, Scherl-Mostageer *et al*. (2001) identified Est sequences corresponding to a cDNA identical to Trask, named CUB domain-containing protein 1 (CDCP1), although the protein product was not identified in this study. Of note, these authors used algorithms that predict a third CUB domain in the extracellular region of Trask; however, in our analysis, we found only two CUB domains and with low homology scores. The proposed third CUB domain falls below the threshold of homology of existing public domain algorithms for protein domain prediction. In addition, CUB domains are often only loosely related at the primary structural level, making sequence-based predictions difficult. The ultimate characterization of the nature of these domains in Trask thus awaits tertiary structural analysis. In another approach to identify tumor-associated proteins, Hooper *et al*. (2003) used subtractive immunization techniques to generate antibodies toward cell surface epitopes preferentially expressed by highly metastatic relative to nonmetastatic carcinomas, and identified a cell surface glycoprotein named SIMA135, identical to Trask, and their analysis identifies two extracellular CUB domains. We have independently cloned Trask as a cell cycle substrate of src kinases with a specific adhesion phenotype and interactions with adhesion proteins. We describe here the full-length protein, its cell cycle expression pattern and its mitotic relocalization, its specific cleavage at Arg^{369} by MT-SP1, its stable interaction and specific cell cycle phosphorylation by src and yes, and its interaction with matrix and adhesion proteins and with MT-SP1. Trask is the first adhesion molecule described that is under cell cycle regulation, and its association and mitotic phosphorylation by src kinases, for the first time, implicates src in the regulation of adhesion in epithelial cells undergoing mitosis.

Proteolytic processing of Trask may be a mechanism of regulation of its function. Trask is expressed predominantly in its cleaved 85 kDa form in MDA-468 breast cancer cells and we see no shifting between cleaved and uncleaved forms during cell cycle progression in these cells (data not shown). However, there are substantial differences in the expression of cleaved and uncleaved Trask among various cancer cell lines, with predominant expression of cleaved Trask in most (not shown). Consistent with this, MDA-468 breast cancer cells, like many other epithelial cancer cell lines, exhibit characteristic upregulation of extracellular proteolytic pathways including activated MT-SP1. MT-SP1, which interacts with and cleaves Trask, is upregulated in many common human cancers including breast, ovarian, prostate, and colon cancers and may be responsible for Trask cleavage in most cancer cells (Bhatt *et al*., 2003; Santin *et al*., 2004). Additional serine proteases may also be able to cleave Trask. The addition of exogenous plasmin to human keratinocytes results in cleavage of gp140 to p80 and this protein is felt to be CDCP1/Trask based on anti-Fak immunoblots that seemingly crossreact with the CDCP1/Trask protein (Brown *et al*., 2004) Although the direct significance of Trask cleavage has not yet been ascertained, it holds promise as an interesting mechanism of regulation of Trask activity.

The interaction of Trask with MT-SP1 may have specific functional implications. MT-SP1 has two extracellular CUB domains that may provide the structural basis for the interaction with Trask, since Trask also has CUB domains and CUB domains are known to be involved in homophilic interactions. MT-SP1 cleaves Trask and releases the N-terminal CUB domain. This would result in the release of binding partners of Trask that interact specifically with the N-terminal CUB domain. This may include adhesion molecules, extracellular peptide growth factors, soluble proteases, extracellular matrix components, matrix metalloproteases, or components of the plasminogen activation cascade. In addition to being a substrate of MT-SP1, Trask may play a role in regulating MT-SP1 activity or in mediating the interaction of MT-SP1 with membrane and matrix proteins or with components of proteolytic cascades. MT-SP1 cleaves a number of cancer-associated proteins. In particular, MT-SP1 cleaves and activates urokinase-type plasminogen activator (uPA), which can trigger the plasminogen activation cascade (Takeuchi *et al*., 2000). Trask may provide a docking surface for MT-SP1 to interact with and activate uPA, leading to juxtamembrane activation of the plasminogen cascade, and increased invasiveness of cancer cells. MT-SP1 also cleaves pro-hepatocyte growth factor (pro-HGF) (Lee *et al*., 2000). Trask may also facilitate this interaction leading to increased HGF signaling, which is associated with increased motility and invasiveness of tumor cells. Similarly, through a potential docking function, Trask may mediate the interaction of MT-SP1 with other soluble proteases involved in matrix remodeling such as matrix metalloproteases.

Trask is clearly a substrate of src family kinases within the cell and its function is likely modulated by phosphorylation of the ICD. Although in MDA-468 cells, Trask interacts predominantly with yes, it is difficult to draw any conclusions with regard to role of individual src family members in regulating Trask or mitotic processes, since src, yes, and fyn have largely overlapping functions. Yes, like src, is also activated in human cancers (Park *et al*., 1993; Pena *et al*., 1995). In

293 cells, Trask interacts equally with both yes and fyn (data not shown). Src kinases are well-established players in oncogenesis, and as more specific inhibitors of these kinases become available, a clearer delineation of the specificity and mechanism of Trask phosphorylation will be ascertained.

Trask is only the second mitotic substrate of src kinases identified to date and the first adhesion molecule found to be under cell cycle regulation. We have shown that Trask is phosphorylated in a cell cycle-specific manner and that Trask represents a novel class of adhesion-related proteins. The phosphorylation of Trask represents a first possible method of regulation. Proteolytic cleavage of Trask is mediated by an extracellular proteolytic system and this may be yet another method of Trask regulation. This dual control and the identification of an adhesion-related, cell cycle substrate of src kinases presents a new paradigm to study the physiologic role of src kinases in mitosis and the pathologic role of src kinases in mediating the invasive and metastatic properties of src-driven tumors.

Materials and methods

Cell culture and immunoblotting

MDA-468 cells were grown in DME:F12 supplemented with 10% fetal bovine serum, glutamine, penicillin, and streptomycin, at 37°C in a humidified incubator with 5% CO_2. For cell cycle synchronization, cells were treated for 20 h with 200 ng/ml nocodazole, 15 μg/ml lovastatin, 5 μg/ml aphidicolin followed by a 2 h release, or 2 μM etoposide for synchronization in M, G1, mid-S, and G2, respectively. The cell cycle phase of synchronized cells was confirmed by FACS analysis of ethidium-stained nuclei measuring DNA content. M cells were further differentiated from G2 cells because of their suspended state, their forward scatter characteristics, and their chromatin condensation. Lysates were prepared in RIPA buffer (10 mM Na phosphate pH 7.2, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% NP-40, 1% Na deoxycholate, and protease inhibitors). Immunoblots were performed using mouse monoclonal antibodies for myc and phosphotyrosine (Santa Cruz Biotechnology), yes (Wako), src (Calbiochem), rabbit polyclonal antibodies for N-cadherin and P-cadherin (Santa Cruz) and MT-SP1 (Takeuchi *et al*., 2000), and goat polyclonal for syndecans 1 and 4 (Santa Cruz). PD173955 and PD179483 were obtained from Parke-Davis Pharmaceuticals. PP1 was from Calbiochem. Anti-Trask monoclonal antibodies were developed against the peptide sequences KERSGVVCQTGRAFMIIQEQRTC and CSPTSGKQLDLLFSV TLTPRT taken from the ECD of Trask.

Purification of Trask

Trask was purified in its hyperphosphorylated state from MDA-468 mitotic lysates. Cells were blocked in mitotis with 200 ng/ml nocodazole and lysed in RIPA buffer. Lysates were boiled for 5 min and used in pilot experiments to screen a panel of anti-phosphotyrosine antibodies for the ability to immunopurify denatured Trask. The experiment was then scaled up and PY20 antibodies were bound to solid support used to immunopurify Trask from denatured mitotic lysates, eluted in phenyl phosphate, and separated by SDS–PAGE.

Amino-acid sequence analysis

The purified gel-bound protein was digested with trypsin, peptides were partially fractionated, and were then analysed by matrix-assisted laser desorption/ionization reflectron time-of-flight (MALDI-reTOF) mass spectrometry (MS) (Erdjument-Bromage *et al*., 1998); matches of the masses were not found within the human segment of a protein nonredundant database. In a parallel analysis, two selected peptides from the pool were sequenced by nano-electrospray ionization (ESI) triple quadrupole MS/MS (Geromanos *et al*., 2000); spectra were inspected for y″ ion series and the information was taken to search dbEST using the PepFrag program. Matches were identified with a potential ORF of a human Est and verified by comparing the computer-generated fragment ion series of the predicted tryptic peptides with the experimental MS/MS data.

Northern blots

PolyA-selected RNA was separated by formaldehyde gel electrophoresis and transferred to a nylon membrane. The membrane was probed with a 2 kb ^{32}P-labeled Trask cDNA probe hybridized in 50% formamide, 5 × SSC, 5 × Denhardt's, 0.1% SDS, 100 μg/ml salmon sperm DNA and washed in 0.2 × SSC, 0.1% SDS at 65°C and exposed to film overnight.

Transfections

Transfections were performed using Lipofectamine 2000 (Invitrogen). MDA-468 cells were stably transfected with pcDNA6/TR (Invitrogen), selected in blasticidin, and expression of the tet repressor confirmed in transient transfection assays. These cells were named MDA468TR. The 2.5 kb human Trask cDNA was cloned by RT–PCR, the sequence was confirmed, and was then ligated into the pcDNA4/TO/MycHis vector (Invitrogen) under control of the CMV promoter containing tet operator sequences. The pcDNA4-Trask vector was stably transfected into MDA468TR cells, and transfectants were selected in Zeocin. In this expression system, doxycycline induces gene expression.

Kinase assays

In vitro kinase reactions were performed in kinase buffer (50 mM PIPES pH 7.0, 10 mM $MnCl_2$, 10 mM DTT, 10 μM ATP, 2 μg acid-denatured enolase, and 5 μCi of γ^{32}P-labeled ATP). Reactions were allowed to proceed at 30°C for 10 min, then immediately stopped by boiling in sample buffer, separated on a 10% SDS–PAGE gel, transferred to membrane, and exposed to film. Src kinase assays were performed using 0.3 μg of purified recombinant src protein (Panvera). Yes kinase assays were performed using yes immunopurified from 300 μg cell lysates using anti-yes monoclonal antibodies (Wako).

Immunofluorescence studies

Cells were grown on glass coverslips, fixed in 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100. Fixed cells were stained initially with anti-Trask 12F3 monoclonal antibodies and subsequently with rhodamine red-conjugated anti-mouse antibodies and counterstained with Hoechst stain. Localization of Trask was studied by confocal microscopy with appropriate filtering.

In vitro *protease reactions*

A 1 μg portion of recombinant $Trask_{ECD}$ was incubated with 100 nM MT-SP1 in PBS for 1 h at 37°C. Laemmli buffer was

added and the sample was boiled for 5 min to stop the cleavage reaction. The resulting sample was run on a 4–20% Tris-glycine SDS–PAGE gel (Invitrogen). The gel was silver stained and immunoblot analysis with anti-Myc antibodies was performed. Complete cleavage of Trask by MT-SP1 was observed. The C-terminal fragment was identified by immunoblot with an anti-Myc antibody (the myc-epitope is on the C-terminus of the protein) and the cleavage products were sequenced by Edman degradation at the UC Davis Molecular Structure Facility.

In vivo *protease inhibition*

MDA-468 cells were grown to 80% confluency and pretreated with the inhibitor ecotin (7 μM) for 2 h. Trask expression was induced with 100 ng/ml doxycycline for 24 h and cell lysates were separated by SDS–PAGE and immunoblotted using anti-myc antibodies.

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