ST14 (suppression of tumorigenicity 14) is a transmembrane serine protease that contains a serine protease catalytic (SP) domain, an SEA domain, two complement subcomponent C1r/s (CUB) domains, and four low density lipoprotein receptor class A domains. Glutathione S-transferase fusion proteins with SP, CUB, and low density lipoprotein receptor domains and their corresponding mutants were generated to analyze protein interactions with these domains. Modified glutathione S-transferase pull-down assays demonstrated the interaction between the SP domain and hepatocyte growth factor activator inhibitor-1. With the same method, a CUB domain-interacting protein was isolated and turned out to be the transmembrane protein with epidermal growth factor-like and two follistatin-like domains 1 (TMEFF1). Quantitative real time PCR revealed that the expression of the TMEFF1 gene was dependent on the transfection of the ST14 gene in the RKO cell line. Our results also suggested that ST14 and TMEFF1 were co-expressed in the human breast cancer cell line MCF7, human placenta, kidney, and liver tissues. Interestingly, these two genes were co-upregulated in kidney tumors versus normal tissues, consistent with our results that showed the dependence of TMEFF1 expression on ST14 in RKO cells. Finally, homology modeling studies suggested that TMEFF1 might form a complex with ST14 by an interaction between epidermal growth factor and CUB domains.

ST14 (suppression of tumorigenicity 14) is a multidomain transmembrane serine protease of the S1 trypsin-like protein family (1). It was first isolated as a novel matrix-degrading protease from human breast cancer cell line by Shi et al. (2). Independently by using subtractive hybridization to isolate genes that are highly expressed in normal intestinal mucosa but not expressed or expressed at a lower level in colon cancers (3), we obtained a clone that is identical to ST14. We further assigned the ST14 gene to human chromosome 11q24 (4). Takeuchi et al. also cloned ST14 from a human prostate cancer cell line and designated it the membrane type serine protease 1 (5). ST14 was detected in colon carcinomas, immunolabeled basement epithelial cell lines, breast cancer cell lines, and prostate cancer cell lines but not in cultured fibroblasts or fibrosarcoma cells (3, 6). The localization of ST14 to the cell membrane was demonstrated by surface biotinylation techniques (7).

ST14 is composed of an N-terminal transmembrane signal, a trypsin-like serine protease catalytic (SP) domain, a SEA domain, two tandem repeats of the complement subcomponent C1r/s (CUB) domains, and four tandem repeats of the low density lipoprotein receptor (LDLR) class A domains (5, 8, 9). Friedrich et al. illustrated the crystal structures of the catalytic domain of ST14 and its complex with a bovine pancreatic trypsin inhibitor (8). Kunitz type serine protease inhibitors, such as hepatocyte growth factor activator inhibitor-1 (HAI-1) (5, 9) and aproatin (10), strongly inhibit ST14 through binding to its catalytic domain. Besides the catalytic domain, other regions are also required for ST14 activation (11). By mutation analysis, the CUB and LDLR domains were shown to be indispensable for the proteolytic activity of ST14 (11). In the present study, we generated GST fusion proteins that express three domains of ST14 and demonstrated the interaction between ST14 and HAI-1 using modified GST pull-down assays. Furthermore, we identified a novel interacting protein, TMEFF1, which binds the CUB domain of ST14. Quantitative real time PCR demonstrated that TMEFF1 was expressed only upon ST14 transfection into the RKO cell line. These two genes were found to be co-expressed in the human breast cancer cell line MCF7, human normal placenta, kidney tumor, normal kidney, liver tumor, normal liver, lung tumor, and breast tumor. Co-up-regulation of these two genes was observed in kidney cancers and remains an interesting phenomenon to be further explored. Finally, we demonstrated a potential complex formation between TMEFF1 and ST14 by using homology modeling approaches.

MATERIALS AND METHODS

Cell Lines and Monoclonal Antibody—Human colon carcinoma cell line RKO was transfected with ST14 in a pSecTag2A vector to establish the RKO-ST14 cell line and was transfected with the pSecTag2A backbone vector to establish the RKO-pSecTag2A cell line, respectively. The SW620-HAI-1 cell line, another human colon carcinoma cell line SW620 transfected with HAI-1, and the human monoclonal anti-HAI-1 antibody were generous gifts from Jingia Ye (12). RKO-pSecTag2A and RKO-ST14 cells were cultured in Dulbecco’s modified Eagle’s medium high glucose medium supplemented with 10% fetal calf serum and 600 μg/ml zeocin at 37 °C in 5% CO2. SW620-HAI-1, the prostate cancer cell line PC3, the human breast cancer cell line MCF7, and Bcap37 were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum in the same condition.

Construction of Expression Vectors for GST-ST14 Domains—Each domain of ST14 was amplified by PCR, ligated into the pGEMTeasy vector (Promega), and subcloned into the prokaryotic expression vector pGEX-4T2 (Amersham Biosciences) by double enzyme digestions with BamHI and EcoRI/NotI (Takara). The resultant constructs were verified by restriction enzyme digestion and DNA sequencing. Primers used for the SP domain were as follows: forward primer, 5’-GGATCC-GACGGGATTAGGCTTCGCTTTG­­TTTGTG-3’ and reverse primer, 5’-GGCGGCGG-
**TABLE 1**

| Primer name | Polarity | Sequence |
|-------------|----------|----------|
| H656A       | Forward  | 5'-TCTTGCCGCACCTGCTCACAT-3' |
| D711A       | Reverse  | 5'-GACGATCAGTTGCAGGAC-3'    |
| S805A       | Reverse  | 5'-GGTGATGCGGGGACCTGTCGTC-3'|
| D482Y       | Reverse  | 5'-TGGCCAAGGGACCTGTCGTC-3'  |
| D519Y       | Reverse  | 5'-GGGACACAGCCAGGACGAC-3'   |
| D555Y       | Reverse  | 5'-GGGGCGCTTAGTCCATGACG-3'  |
| D598Y       | Reverse  | 5'-GTGACGGCTCTGACTGACA-3'   |

*Lowercase letters in the primer sequence represent changed nucleotides.*

**Protein Interactions of ST14 Domains**

GST Pull-down Assays with Columns—To compare the possibility of protein interaction between the wild-type and mutant forms of GST-ST14 domains, modified GST pull-down assays were used with glutathione-Sepharose 4B columns as described (16). GST fusion proteins and their corresponding mutants were immobilized onto glutathione-Sepharose 4B columns, respectively. The whole cell lysates were loaded onto columns and incubated overnight at 4 °C. The columns were then washed thoroughly with PBS to minimize the contamination. Finally, saline gradient elutions with a 5-column volume of PBS with NaCl concentrations of 200, 400, 600, 800, 1000, and 2000 mM were performed sequentially. Amicon Ultra-4 centrifugal filter units (Millipore Corp.) with a 5000-Da molecular mass cut-off were used to concentrate each elution product to 50 μL. The proteins bound to GST fusion proteins or their mutants were analyzed by 10% SDS-PAGE with Coomassie Blue staining.

Peptide Mass Fingerprint Analysis—Protein bands of interest were excised from Coomassie Blue-stained gel and cut into small pieces. After being removed of SDS, gel pieces were digested by sequencing grade modified trypsin (Promega) at 37 °C overnight. The peptides extracted from the gel were analyzed by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (ProteinChip System (Ciphergen) according to the manufacturer's protocol.

Western Blot Analysis—Protein concentration was measured by the DC Protein Assay Kit (Bio-Rad). Equal amounts of proteins were resolved by 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Bio-Rad). The membrane was first blocked in TBST (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, and 0.1% Tween 20) with 5% nonfat dry milk and then probed with primary antibody (monoclonal anti-HAI-1 antibody, 1:1000 in TBST with 5% nonfat dry milk), followed by incubation with secondary antibody (horseradish peroxidase-conjugated antibodies; 1:5000 in TBST with 5% nonfat dry milk). The results were visualized by using the Lumi-Light Western blotting substrate (Roche Applied Science).

Quantitative Real Time PCR—Total RNA was isolated from cell lines or tissues using the RNeasy Mini Kit (Qiagen). For each reverse transcription-PCR, 2 μg of total RNA was converted into cDNA using Moloney murine leukemia virus reverse transcriptase (Promega). Two microliters of reverse transcription reaction was then quantified by quantitative real time PCR using an ABI PRISM 7900 Sequence Detector and ABI TaqMan® PCR Master Mix (Applied Biosystems). The quantitative results of ST14 and TMEFF1 mRNA were normalized to β-actin mRNA from the same sample. Primers used for quantitative real time PCR were synthesized at GeneCore Corp. Primers used for ST14 were as follows: forward primer, 5'-TGGCTACATGAGGATCGCAAAATG-3'; reverse primer, 5'-AGGATGGGACTCCGCTGTAC-3'. The TaqMan® probe used for ST14 was 5'-FAM-TCGCTAGTGGGCCTGCACAT-3'. The TaqMan® probe used for TMEFF1 was as follows: forward primer, 5'-AATGTGAGGAGGTCTGACGTAGGAG-3'; reverse primer, 5'-TCCCCATTTGTACCCAGACA-3'. The TaqMan® probe used for TMEFF1 was as follows: forward primer, 5'-FAM- TGTCGACCTTGGCGGGATGG-3'; reverse primer, 5'-TCCCCATTTGTACCCAGACA-3'. The TaqMan® probe used for TMEFF1 was as follows: forward primer, 5'-FAM-TGGCTACATGAGGATCGCAAAATG-3'; reverse primer, 5'-AGGATGGGACTCCGCTGTAC-3'.

**Structure Modeling**—The query protein sequence was submitted to the metaserver @TOME (17) and analyzed by six different fold recognition or protein structure prediction servers, including PDB-BlasT (18), 3D-PSSM (19), mGenTHREADER (20), FUGUE (21), Sam-T99 (22), and Jpred2 (23). After the three-dimensional compatibility
between the submitted sequence and the retrieved Protein Data Bank templates was evaluated, models were built directly by using MOD-ELLER (24) with the top ranking structural alignments. Predicted models were superimposed to the coordinates in the retrieved templates using Swiss-PdbViewer (25).

RESULTS

Expression and Purification of GST-ST14 Domain Fusion Proteins and Their Mutants—GST-ST14 domain fusion proteins were designed essentially as described by Lin and co-workers (11) and are illustrated in Fig. 1. Mutations that substitute His-656, Asp-711, or Ser-805 with Ala in the SP domain would inactivate the serine protease activity (8). Deletion of the second CUB domain would result in a poor activation of ST14 (11). The point mutations that substitute Asp with Tyr in each of the four LDLR domains would inactivate the Ca$^{2+}$-binding cage by substituting Asp-598, Asp-555, Asp-519, and Asp-482 with Tyr (as shown in GST-mutLDLR). GST-CUB contains two CUB domains, and GST-delCUB contains only the first domain. TM, transmembrane domain.

Enzyme Activity Assay of GST-SP Fusion Proteins and Mutants—We diluted purified GST-SP and GST-mutSP fusion proteins to the same concentration and tested their enzyme activities in parallel. GST-SP fusion proteins following incubation with aprotinin in a final concentration of 5 μg/ml were tested as control. Aprotinin shows strong inhibitory activity on serine protease activity of ST14 (10). As illustrated in Fig. 4, aprotinin inhibited the activity of purified GST-SP fusion proteins completely (Fig. 4, curve 3). Similarly, no serine protease activity was detected in the GST-mutSP fusion proteins that were mutated in the catalytic triad of the SP domain (Fig. 4, curve 2). Purified GST-SP fusion proteins were autoactivated slowly, and this activity lasted for several days (Fig. 4, curve 1).

Detection of the Interaction between GST-SP and HAI-1 by GST Pull-down Assays—To confirm the interaction between the SP domain and HAI-1, GST-SP fusion proteins were immobilized onto the glutathione-Sepharose 4B beads and tested for their ability to pull down the HAI-1, GST-SP fusion proteins were immobilized onto the glutathione-Sepharose 4B (lane 3), whereas autoactivation was more obvious in the whole cell lysate (Fig. 2, lane 3). Similar autoactivation was reported when a His-805 (as shown in GST-mutSP) was present in the SP domain would inactivate the serine protease activity (8). Deletion of the second CUB domain would result in a poor activation of ST14 (11).

FIGURE 1. Mutations that substitute His-656, Asp-711, or Ser-805 with Ala in the SP domain would inactivate the serine protease activity (8). Deletion of the second CUB domain would result in a poor activation of ST14 (11). The point mutations that substitute Asp with Tyr in each of the four LDLR domains would inactivate the Ca$^{2+}$-binding cage by substituting Asp-598, Asp-555, Asp-519, and Asp-482 with Tyr (as shown in GST-mutLDLR). GST-CUB contains two CUB domains, and GST-delCUB contains only the first domain. TM, transmembrane domain.
Protein Interactions of ST14 Domains

GST-ST14 Domains as Bait to Capture Prey Proteins—Three cell lines, including Bcap37, RKO-pSecTag2A, and RKO-ST14, were used as sources of potential ST14-interacting proteins. ST14 is detectable in Bcap37 but not in RKO. Therefore, a full-length cDNA of ST14 was cloned into pSecTag2A and transfected into RKO cells. RKO-pSecTag2A was the RKO cell line transfected with the empty pSecTag2A vector and used as a control. The reverse transcription-PCR confirmed the successful transfection. Western blot analysis indicated that HAI-1 was not detectable in these cell lines (Fig. 6A).

Three pairs of GST fusion proteins (GST-SP, GST-CUB, and GST-ST14) along with their mutants were used to capture their interacting proteins in modified GST pull-down assays. Either the proteins bound to both wild-type and mutant ST domains, or bound proteins not sufficient for identification were excluded from the candidate pool of ST14-interacting proteins. The protein bands that were present in the fraction bound to the wild-type domains of ST14 but not in that bound to their mutants were excised from the SDS-polyacrylamide gel for peptide mass fingerprint analysis. No such specific binding proteins were identified in the assays of SP and LDLR domains. However, we obtained a protein of ~37.6 kDa that was still bound specifically to the wild-type CUB domain in the buffer with 800 mM NaCl (Fig. 6B). This 37.6-kDa protein was detected only in the RKO-ST14 cells, and it was not the GST or GST fusion protein according to its molecular weight. Interestingly, this protein was not detected in Bcap37 or RKO-pSecTag2A cells.

Peptide mass fingerprint analysis of this protein was shown in Fig. 7. By searching a peptide mass fingerprint data base (Aldente; available on the World Wide Web at au.expasy.org/tools/aldente/), this protein was TMEFF1. This result further indicated that this protein is TMEFF1, although it remains unknown whether this protein is the processed form of TMEFF1 or its precursor.

Co-expression of ST14 and TMEFF1 in Cell Lines and Tissues—Human placental tissue and a breast cell line MCF7 expressed ST14 and TMEFF1 at different levels. Northern blot analysis showed TMEFF1 was expressed at a moderately low level in the prostate cancer cell line PC3 (27). The ST14 mRNA is undetectable in PC3 according to quantitative real time PCR results of Bhatt et al. (28), although Takeuchi et al. (5) cloned ST14 cDNA from PC3 cells. Thus, we chose placental tissue, MCF7, and PC3 cell lines in our study. As shown in Fig. 8, our results confirmed the co-expression of ST14 and TMEFF1 in human placental tissue. However, ST14 was expressed at a low level in MCF cells and was
undetectable in PC3 cells. We further tested the expression of ST14 and TMEFF1 in the tumor and matched adjacent normal tissue of kidney, liver, lung, breast, and colon. Co-expression of ST14 and TMEFF1 was identified in normal kidney, kidney tumor, normal liver, liver tumor, lung tumor, and breast tumor. Interestingly, ST14 and TMEFF1 were co-up-regulated in kidney tumors versus normal tissues. N, normal; T, tumor.

**DISCUSSION**

ST14 is an interesting multidomain protein, which has been implicated in cancer metastasis (29, 30). Different research groups have reported different splicing variant forms of ST14 since 1993. As shown in Table 2, five forms of ST14 have been detected with a molecular mass of 95, 80, 70, 45, and 25 kDa, respectively. It is postulated that these five different forms of ST14 are composed of the full-length protein (with a calculated molecular mass of 95 kDa), residues 149–855 (78 kDa), residues 190–855 (73 kDa) or 205–855 (74 kDa), residues 190–614 (45.7 kDa), and residues 615–855 (26 kDa), respectively. These different forms of ST14, which contain various domains, are conceived to play different roles in different subcellular locations. Some forms of ST14 localize to the cell membrane, and others are secreted (7, 9, 11, 31).

The SP domain is important for the activation of hepatocyte growth factor and urokinase-type plasminogen activator. HAI-1, aprotinin, and eotin inhibit the serine protease activity of ST14 by binding to this domain (5, 10). The CUB and LDLR domains have been thought to be involved in protein-protein interactions or protein-ligand interactions (32, 33), but no interactions were reported for these domains on ST14. The CUB domain was originally identified in the complement subcomponents C1s and C1r (32). Most of the CUB domain-containing proteins are involved in development processes (32). The combinations of CUB with a variety of other protein domains have been implicated in two biochemical functions: (a) protein or ligand binding when the CUB domain is combined with either EGF, LDLR, Sushi, FA58C, Speract, or MAM domains and (b) proteolytic activity when the CUB domain is present in proteins such as astatin and trypsin-serine protease (34).

In the present work, three pairs of wild-type and mutant forms of GST ST14 domain fusion proteins were used to investigate the interacting proteins of each domain of ST14. We identified a CUB interacting protein, TMEFF1. Our results suggest that a CUB domain might interact with the EGF domain of TMEFF1 and form a complex with the EGF domain similar to the CUB1-EGF-CUB2 domain of Masp2.

TMEFF1 is a transmembrane protein, which contains a unique EGF domain and two Kazal_1 domains (35). These domains implicate a role for TMEFF1 in growth factor signaling (27). Several proteins that participate in the regulation of growth factor signaling, CSF1, IGF2, and TGFα have also been detected at high levels in the cell line RKO-ST14 (36). It has been suggested that TMEFF1 inhibits nodal signaling through binding to the nodal co-receptor Cripto (37). Although the Kazal_1 domain is known to inhibit a number of serine peptidases of the S1 family (35) that includes ST14 (38), Yamashita et al. (10) showed that pancreatic secretory trypsin inhibitor containing a Kazal_1 domain did not inhibit ST14. Similarly, the GST pull-down assays in our work also did not provide evidence to support the interaction between the SP domain and TMEFF1.

The interaction of ST14 and TMEFF1 was not yet verified directly in our study because of the difficulty in expressing GST-ST14 (about 117 kDa) in E. coli. Nonetheless, our current results have strongly suggested the possibility that TMEFF1 participates in the ST14 activation through binding to the CUB domain. Protein processing and activation of ST14 are important for its biological role. As shown in Table 2, ST14 can be processed at residues 615, 205, 190, and 149 (11). We conceive that a group of regulators (activators and inhibitors) is necessary to determine the ST14 activation in a timely and spatial fashion by interacting with ST14. It is likely that HAI-1 and TMEFF1 govern different steps of ST14 activation, respectively. Additionally, the fact that ST14 and TMEFF1
co-localize on the cell membrane surface (27, 39) also supports the possible interaction, since mature ST14 is secreted into human milk in a complexed form (9).

Northern blot analysis has revealed that TMEFF1 is expressed predominantly in the brain. Interestingly, matriptase-3, which is 31% identical to ST14, is also expressed at a high level in the brain (40). The expression of TMEFF1 is down-regulated in brain tumors (27), but the relationship between matriptase-3, TMEFF1, and cancer diseases has not been established. ST14 and TMEFF1 are hypothesized as tumor suppressor genes in colon cancers and in brain cancers, respectively (3, 27, 29, 36, 41). ST14 was detected, however, at very low levels in normal tissues but at high levels in breast carcinomas (6, 42). In the present work, co-expression of ST14 and TMEFF1 was identified in human placenta, kidney, liver tissues, and MCF7 cells. More interestingly, the association between the expressions of these two genes was suggested both in vitro in cultured RKO-ST14 cell lines and in vivo in kidney tumors and normal kidney tissues. Co-up-regulation of ST14 and TMEFF1 in kidney tumors is not necessarily in conflict with the hypothesis that TMEFF1 behaves as a tumor suppressor gene in brain cancers. Take together, various expression levels in different tissue sites suggest tissue-specific roles of ST14 and TMEFF1 and their interaction in tumorigenesis, although more clinical samples need to be examined for this hypothesis.

The GST pull-down assay is a classical method to identify protein-protein interactions in vitro. In a typical pull-down assay, a GST fusion bait protein is bound to an immobilized glutathione support and used to detect prey proteins from cell lysates. The interacting proteins are then detected by SDS-PAGE or Western blot. Various washing steps have been described (14, 15, 43), and a universal protocol does not exist. Our modified GST pull-down assay has improved the classical assay in the situation that the bait protein differs not very much in binding to its partners of ST14 domains.

Serine protease activity of GST-SP was verified using tissue plasminogen activator chromogenic substrate. Since the prokaryotic expressing system lacks SP activation machinery, this process may be an autoactivation. It is consistent with the report of Takeuchi et al. (5), in which a His-tagged fusion protein of ST14 was expressed in E. coli. The biological function of SP autoactivation needs further attention.

As shown in Table 2, different forms of ST14 are detected in different cell lines and tissues. They have different molecular weights, cell localization, and protein partners. Further exploration of the wild-type ST14 in its different cellular forms and its corresponding mutants is currently being carried out to uncover the roles of these different forms of ST14. Such a study will be of great help for our understanding of the membrane-associated proteolytic system and the mechanism of cancer metastasis.

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TABLE 2
Different cellular forms of ST14 reported in the literature

| Year | Source | Molecular mass | Components | N-terminal sequencing | Reference |
|------|--------|----------------|------------|-----------------------|-----------|
| 1993 | Human breast cancer cells | 80 | NA* | | 2 |
| 1997 | T47D (cell line) | 80 | 40 kDa | NA | 7 |
| 1997 |  | 95 | 80 kDa | NA | 7 |
| 1999 | Human milk | 95 | A: ST14 | NA | 9 |
| 1999 |  | 110 | B: 40 kDa (HAI-1) | GPPAPPGEPG (HAI-1: 1) | 9 |
| 1999 |  | 110 | C: 25 kDa (partial HAI-1) | TQGFEGS (HAI-1: 154) | 9 |
| 1999 | COS-7 (transfected cell line) | 95 | ST14 | NA | 1 |
| 1999 | E. coli (transfected) | 52 | The SP domain of ST14 | VSVGTT (ST14: 615) | 5 |
| 2001 | Human milk | 95 | A: 70 kDa; A-1: 45 kDa | SFVVT (ST14: 190) or TVQRT (ST14: 205) | 31 |
| 2001 |  | 120 | B: 40 kDa (HAI-1) | VSVGTT (ST14: 615) | 31 |
| 2001 | T46D (cell line) | 70 | NA | NA | 31 |
| 2001 | BT549 (transfected cell line) | 70 | Processed ST14 | NA | 11 |
| 2001 |  | 120 | Complexed with HAI-1 | NA | 11 |
| 2004 | Bcap37, Colo205, and SW480 (cell line) | 95 | Glycosylated ST14 | NA | 44 |
| 2004 |  | 60 | ST14 | NA | 44 |
| 2005 | SW620 (cell line) | 60 | ST14 | NA | 44 |
| 2005 | Bcap37 (transfected cell line) | 120 | Glycosylated ST14 | NA | 45 |
| 2005 |  | 75 | ST14 | NA | 45 |

* NA, not available.

a Position number of the residue where the N-terminal sequence starts.

b NA, not available.
Protein Interactions of ST14 Domains