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An Interaction Between Apolipoprotein E and TERE1 With a Possible Association With Bladder Tumor Formation

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Abstract TERE1, a recently discovered gene/protein appears to play a role in bladder tumor growth regulation but to date does not have clear functional correlates. The objective of this study was to gain further insight into the function of the TERE1 protein by identifying potential protein to protein interactions with TERE1 and determining whether these interactions are associated with putative growth regulatory pathways and/or bladder tumor formation. Towards this aim, we have performed a bacterial two hybrid assay and isolated interacting clones, which then were sequenced and further examined by affinity chromatography and immunoprecipitation. From among several positive clones, we isolated a putative interacting plasmid containing the C-terminal portion of preapolipoprotein E starting from amino acid number 124 from the pBT-TERE1/pTarget-cDNA bacterial two hybrid system. The C-terminal portion of apoE interaction with the TERE1 was confirmed using ProBond columns by the expression of 6XHis recombinant and ^{35}S methionine/cysteine labeled proteins. We found that there was ubiquitous expression of the apoE transcript in normal bladder and in various grades and stages of transitional cell carcinoma (TCC) of the bladder. Likewise, we detected the apoE protein in both normal and malignant bladder tissues by Western blot. There was a significant decrease in the apoE protein in 12 of 16 muscle invasive TCCs of the bladder compared to normal bladder mucosa samples. Previous studies in rat fibroblasts have found that expression of apoE can decrease the phosphorylation of the growth factor-related p42/44 MAP kinase. A significant decrease in p44/p42 MAPK phophorylation was also apparent using a phosphorylation specific antibody in human 293 kidney cells upon transfection and expression of apoE. In conclusion, the results from this study suggest that the expression and regulation of the apoE pathway may yield clues toward understanding the function of TERE1. J. Cell. Biochem. 95: 419–428, 2005.

Transitional cell carcinomas (TCCs) of the bladder is the fifth most common cancer in the United States and accounts for 54,300 new cases and 12,400 cancer related deaths per year [Jemal et al., 2003]. Approximately 25%–35% of new cases of TCC present with muscle invasion at the time of diagnosis [Catalona, 1988; Malkowicz and Wein, 1994]. While various genetic alterations have been associated with advanced TCC, the perturbations in these genes do not completely explain the progression of TCC to muscle invasive bladder cancer [Aveyard et al., 1999; Koo et al., 1999; Bohm et al., 2002; Lu et al., 2002; Sun and Herrera, 2002; Urist et al., 2002; Adachi et al., 2003; Chan et al., 2003; Chang et al., 2003; Hoque et al., 2003; Hussain et al., 2003; Karashima et al., 2003; Ohnishi et al., 2003; Wu et al., 2003].

We have recently identified a novel cDNA product designated transitional epithelial response gene (*TERE1*), which has a potential role in the progression of bladder cancer [McGarvey et al., 2001]. The *TERE1* gene has been localized to chromosome 1p36 and the TERE1 transcript (1.5 and 3.5 kb) is generally expressed in normal human tissues including urothelium. Interestingly, allelic loss in on chromosome 1p was detected in higher stage bladder cancers [Hoque

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et al., 2003]. The open reading frame encodes a protein of 338 amino acids (molecular weight 36.8 kD). This protein is not homologous to any known human protein, but is homologous to a *Drosophila* protein designated *heix*, which appears to be important for growth control during fly development.

While, we have not identified any *TERE1* mutations, the TERE1 transcript was found to be under-expressed in the majority of muscle invasive TCC tumors (22 out of 29 cases) studied. Using a polyclonal antibody to a specific TERE1 peptide, we have shown by Western blotting and immunohistochemistry, a decreased or absent staining in 42% of muscle invasive tumors (McGarvey et al., unpublished data). Transfection of a sense TERE1 construct resulted in an 80%–90% inhibition of cellular proliferation in two TERE1 under-expressing TCC cell lines. Retroviral transduction of TERE1 also reduced proliferation with an apparent increase in genomic stability. These data suggest a potential role for this newly identified gene product in the progression of bladder cancer [McGarvey et al., 2001]. The aim of this investigation was to determine TERE1 protein to protein interactions in order to further examine the function of TERE1.

MATERIALS AND METHODS

Bacterial Two Hybrid System

The BacterioMatch Two-Hybrid system is based on methodology developed by Shaywitz et al. [2000]. The technique uses transcriptional activation (Stratagene, Inc., La Jolla, CA). The TERE1 ORF was amplified using gene specific primers including the EcoR1 and Xho1 restriction enzyme recognition sites and then cloned into the EcoR1/Xho1 sites of the pBT vector, which includes full-length bacteriophage λcI protein containing the amino-terminal DNA-binding domain and the carboxyl-terminal dimerization domain. The TERE1 ORF was then sequenced and found to be in frame with the bacteriophage λcI protein. The bait (TERE1) is tethered to the λ operator sequence upstream of the reporter promoter through the DNA-binding domain of λcI. The corresponding target cDNAs (from a human liver cDNA library) had been cloned downstream of the N-terminal domain of the α-subunit of RNA polymerase in the pTRG vector (Stratagene, Inc.). When the bait and target interact, they recruit and stabilize the binding of RNA polymerase close to the promoter and activate the transcription of a reporter gene (Ampicillin). Co-transformation was performed on the BacterioMatch Two-Hybrid system reporter strain competent cells with the purified TERE1-pBT vector and human liver cDNA library-pTRG and initial screening took place in the presence of chloramphenicol, tetracycline, kanamycin, and carbenicillin. A second reporter gene, β-galactosidase, is expressed from the second activatable promoter to allow secondary screening in the presence of X-gal and an X-gal inhibitor.

Prokaryote Expression of TERE1

The open reading frame of TERE1 was amplified by RT/PCR from normal bladder mucosa and cloned into the pCRT7/NT TOPO expression vector (Invitrogen, Inc., Carlsbad, CA) according to the manufacturer's instructions using the following primers specific for the TERE1 open reading frame (301–1446) (Tere1 sense 5′-GGAGCTTCCAT GGCGGCCTCTC-3′, and Tere1 antisense 5′-GATCTCGAGGCAGG-AGTTCC CACCCATGCTTAG-3′). The pCRT7/NT-TERE plasmid was isolated by standard alkaline mini-prep procedures and then used to transform BL21(DE3)pLysS cells. Colonies grown on ampicillin and chloramphenicol plates were picked, inoculated into 10 ml of LB (+ampicillin and chloramphenicol), and grown overnight. The expression of the *TERE1* gene was induced by IPTG (1 mM) the following day over a 4–6 h time period. Samples were boiled in NuPAGE SDS buffer, electrophoresed on NuPAGE gels (Invitrogen, Inc.), and gels were stained with Coomassie Blue.

The 6XHis/TERE fusion protein was purified from pCRT7/NT-TERE transformed BL21(DE3)pLysS bacterial lysate in its native conformation on ProBond purification modules according to the manufacturer's instructions (Invitrogen, Inc.).

HisC Fusion Protein Protocol

The C-terminal portion of the apoE transcript was cloned in frame into the pcDNA3.1/His C vector (Invitrogen, Inc.) by cutting the HisC and apoE/pTarget vectors with the EcoR1/XhoI restriction enzymes (NE Biolabs, Beverly, MA). After overnight ligation, the product used to transform competent *E. coli* (strain JM109, Promega, Inc., Madison, WI). Standard mini-preps were performed on clones, select isolated

plasmid preps restriction enzyme digested, and the clone containing the apoE open reading frame was sequenced to confirm that the 6XHis domain and apoE sequence were in frame. The His/apoE fusion protein was purified from 6XHis/apoE transformed BL21 bacterial lysate on ProBond purification modules (Invitrogen, Inc.). B21-CodonPlus competent cells were purchased from Stratagene, Inc. The recombinant 6XHis/C-terminal apoE fusion protein lysate was reduced by boiling in running buffer, electrophoresed on PAGE, blotted onto nitrocellulose, and detected with a specific antibody to 6XHis (Invitrogen, Inc.) and to apoE (Santa Cruz Biotechnology, Santa Cruz, CA).

In Vitro Transcription/Translation Protocol

The ORF of TERE1 was cloned into the EcoR1/Sal1 sites of the pTNT plasmid (Promega, Inc.), which contains a T7/Sp6 promoter and a 5′ beta-globin leader sequence. The TERE1 open reading frame was obtained by RT/PCR using the sense primer from 308 bp, containing additional Kozak (italic) and EcoR1 lead sequences (5′-CGG/AATTC*GCCA***CCATGGCGGCCTCTCAGG)** and antisense primer from 1412 site, containing the Sal1 lead sequence (5′-ATCG/TCGAC**GCCAAAT CACATTCTTCCTCA**-3′). ^{35}S methionine and cysteine (Applied Bioscience, Foster City, CA) labeled TERE1 was produced by in vitro transcription/translation using the TnT coupled reticulocyte lysate system according to the manufacturer's instructions (Promega, Inc.). Alternatively, EcoR1/Xho1 excised, C-terminal portion of apoE was ligated to the pGEM-11 plasmid, and ^{35}S methionine and ^{35}S cysteine labeled apoE was produced as above. The full-length apoE plasmid was also used as a template for the in vitro transcription/translation assay.

Binding Assay Protocol

Protein extracted from BL21 cells transfected with 6XHis/LacZ (negative control) or 6XHis/apoE was incubated with 50 μl ProBond beads for 1 hr at 4°C. The beads were washed with PBS, non-specific sites blocked with PBS + 1% Triton X-100 + 1% BSA for 1 hr at 4°C, and then incubated for 2 hr at 4°C with ^{35}S methionine/cysteine labeled TERE1. The beads were washed five times with PBS + 1% Triton X-100, boiled in running buffer, and run on PAGE. The TERE1 protein was then detected after drying the gel and exposure to X-ray film. Alternatively, 6XHis/TERE1 was incubated with the ProBond resin followed by incubation with ^{35}S methionine/cysteine labeled C-terminal apoE, and treated as above.

Reverse Transcription-Polymerase Chain Reaction

Total RNA was extracted from tissue culture grown tumor cells (including six TCC, two breast carcinoma, one neuroblastoma, and one prostate carcinoma cell lines) or from frozen normal and malignant bladder tissue by the modified single-step method using Tri-reagent or RNeasy mini kit (Qiagen, Inc., Valencia, CA). Total RNA was isolated from micro-dissected normal human bladder mucosa, normal kidney and various bladder and renal cell carcinoma samples with no more than 20% normal tissue. A master mix for reverse transcription was prepared containing an oligo $d(T)_{16}$ (2.5 μM) along with less than 1 μg of total RNA, and incubated at room temperature for 10 min then at 42°C for 1 h. The reaction was heated at 95°C to destroy reverse transcriptase activity and then cooled to 4°C. For each sample, a PCR master mix of 40 μl was prepared, containing 0.5 μl of AmpliTaq DNA polymerase, and one 0.15 μM of each primer pair, and added to 0–10 μl of the cDNA. Twenty-five to 35 cycles of PCR was performed with a final extension step of 72°C for 10 min. The annealing temperature was dependent on the melting temperatures of each primer pair. The PCR products were run on a 1.2% agarose gel. PCR products were excised from the gel and purified away from the agarose using the Qiaquick gel extraction kit (Qiagen, Inc.) for sequencing.

Each cDNA was used as a PCR template using either set of apoE specific primer between bp 30–452 (sense 5′-AGGAGCCGACTGGCCAATC-3′, and antisense 5′-CCGCACACGTCCTCCATGTC-3′) and bp 475–99 (sense 5′-GAGGTGCAGGCCATG CTC-3′, and antisense 5′-TTGAGGCGGGCCTGG AAG-3′).

Western Blot and Immunodetection

Total protein from TCC tumor cells, 293 human embryonic kidney, normal human bladder and kidney as well as various bladder and renal cell carcinoma tumor tissue samples was extracted in RPI buffer [PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS plus proteinase inhibitors (10 mg/ml PMSF, 30 μl/ml aprotinin,

and 100 mM sodium orthovanadate)]. Protein concentrations were determined based on the Bradford method using BSA as the standard (BioRad Laboratories, Hercules, CA). Thirty microgram of total protein was separated on a 6% SDS-polyacrylamide gel (Tris-glycine) and the separated proteins transferred to a nitrocellulose membrane. The membrane was blocked in PBS containing 5% dried nonfat milk and 0.1% Tween 20 and then probed for 1 hr with a apoE specific monoclonal antibody (A1.4) (Santa Cruz Biotechnology). After washing, the membrane was incubated with a perioxidase conjugated secondary antibody for rabbit Ig raised in donkey (Amersham Pharmacia Biotech, Piscataway, NJ) for 1 hr and the apoE protein detected using ECL detection system (Pierce Biotechnology, Rockford, IL).

MAP kinase (p44/p42) and phopho-p44/42 MAP kinase were immuno-detected from control transfected and ApoE transfected 293 cell line (24 and 48 hr) using p42 MAP kinase and phosphor-p44/42 MAP kinase specific antibodies (Cell Signaling Technology, Inc., Beverly, MA).

Far Western Analysis

First, under denaturing conditions, proteins extracts from normal human bladder mucosa and four bladder tumors were resolved on a 4%–20% gradient NuPAGE gel and then transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk + TBST and incubated with purified in vitro translated 6XHis-TERE1 or 6X-apoE overnight at 4°C. Membranes were extensively washed in TBST, and detected carried out with the Xpress primary antibody (Invitrogen, Inc.). Purified in vitro 6XHis-LacZ protein was used a negative control.

Tissue Culture and Administration of Recombinant apoE to TCC Cell Lines

Various bladder tumor cells lines were obtained from ATCC (Manassas, VA) and cultured according to standard protocols. Serum-free conditioned media obtained from apoE transfected 293 cells was concentrated by dialysis, the apoE concentration estimated by serial dilution on a Coomassie Blue stained SDS–PAGE [compared to a known concentration of recombinant apoE (Sigma Chemical, St. Louis, MO)], and applied to TCC cells for 1, 3, and 6 h. Control cultures were treated with conditioned media from 293 cells transfected with the HisC-LacZ vector. Proteins lysates were electrophoresed on NuPAGE gels (Invitrogen, Inc./Novex) and Western blotting performed using antibodies to pMAP kinase (p42/p44).

RESULTS

We isolated seven distinct of TERE1 interacting clones using the BacterioMatch Two-Hybrid system (Table I). Upon clone sequencing, we found that two positive clones contained a pTarget plasmid with the C-terminal portion of the preapolipoprotein E sequence starting from amino acid number 124–317. We then cloned the C-terminal portion of apoE into the EcoR1/Xho1 sites of the pcDNA3.1HisC vector. After transformation, expression of the *apoE* gene was induced by IPTG (1 mM) in BL21(DE3)pLysS cells. Likewise, the open reading frame of TERE1 was amplified by RT/

TABLE I. TERE1 Interacting Proteins

Gene Name	Accession no.	Functional Pathway
1. *TC1RG1* (TIRC7)	NM006053	T-cell activation [Utku et al., 1998] Vacuolar H+-ATPase [Li et al., 1996]
2. Alpha antitrypsin	X016833	
3. Preapolipoprotein E	AF261279	Inhibits serum-stimulated proliferation
4. GDP-4-keto-6-deoxy-D-mannose epimerase reductase (FX protein)	BC001941	Tissue specific transplantation
	NM003313	Antigen P35B [Tonetti et al., 1996]
5. Transducin (beta)-like 2 (TBL2) isoform 1	NM012435/AF056183	Nuclear signaling pathway
	AF097484/AF097485	Nuclear localization signal
6. JM5 protein	BC003037/BC000464	Xp11.23 transcript similar to XP010204 (with a WD40 repeat)
7. XPC AS EST	AI702437/BI520554 AI452675/BQ067419 AA161242/AW772514	

PCR from normal bladder mucosa and cloned into the EcoR1/Sal1 sites of the pTNT plasmid vector for in vitro transcriptional/translation, followed by a 6XHis-apoE/rTERE1 binding assay. The presence of the ^{35}S labeled TERE1 in the elution confirms the interaction of C-terminal portion apoE and TERE1 (Fig. 1). Using the alternative binding assay (6XHis-tagged TERE1 + in vitro transcribed/translated apoE) allowed for the detection of the ^{35}S-labeled C-terminal portion of apoE (data not shown).

TERE1 polyclonal antibody, which was produced from a TERE1 specific peptide, was used to perform a series of immunoprecipitations. TERE1 coprecipitated with a variety of proteins of various molecular weights ranging from ~200, 125, 110, 50, 42, 35, and 32 kD in lysates of ^{35}S-methionine-labeled 1376 TCC cells (Fig. 2A). The pattern of immunoprecipitated proteins using a distinct primary antibody raised against a peptide from the KIAA1096 protein was different than those proteins using the TERE1 polyclonal antibody. Cell-free translation indicates that the apoE product has an Mr = 28,500 that is glycosylated. In addition, a Western blot of an SDS–PAGE containing proteins immunoprecipitated was performed with the monoclonal antibody to apoE and from normal bladder and kidney and then detected with the polyclonal TERE1 antibody and a HRP-labeled anti-rabbit IgG (Fig. 2B). While these immunoprecipitations are not confirmatory, the immunoprecipitated proteins are at the correct molecular weight for ApoE and TBL2.

cDNA derived from various tumor cell lines and from frozen human tissue (normal human bladder mucosa and 20 bladder tumors) was used as a PCR template using apoE specific primers. We found that all tumor cell lines and tissues examined expressed the ApoE transcript (Fig. 3).

Immunoblotting was performed using an apoE specific antibody on total protein derived from a number of TCC tumor cells, the 293 human embryonic kidney cell line, normal human bladder and kidney, as well as bladder and renal cell carcinoma tumor tissue samples. We found that there was a decrease in ApoE protein levels in the TCC and RCC samples as compared to normal human bladder mucosa and normal human kidney (Fig. 4A,C). We quantitated the levels of apoE protein in 30 cases of TCC, and found a significant decrease in ApoE protein levels in 12 of 16 invasive TCC cases ($P < 0.0001$). Interestingly, there was no detectable expression of apoE in the various TCC and other tumor cell lines except in the SY neuroblastoma cell line (Fig. 4B). We did detect the expression of the apoE protein in the UMUC-3 bladder cell line (data not shown).

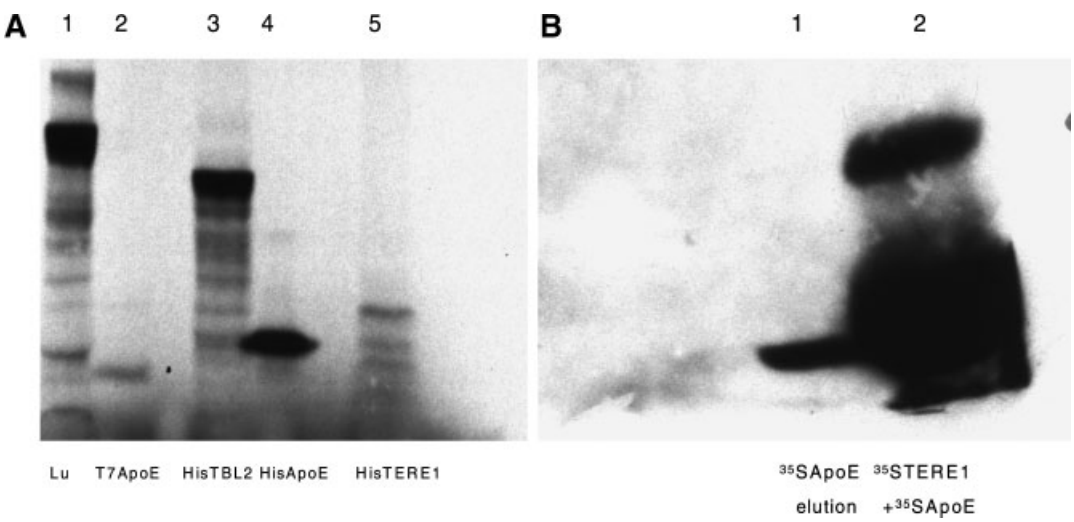

Fig. 1. **A**: In vitro transcription/translation. ^{35}S methionine and cysteine labeled luciferase (**lane 1**), T7truncated ApoE (**lane 2**), 6XHis-TBL2 (**lane 3**), 6XHis-truncated ApoE (**lane 4**), and 6X-HisTERE1 (**lane 5**) were produced by in vitro transcription/translation using the TnT coupled reticulocyte lysate system according to the manufacturer's instructions (Promega, Inc.). The molecular weight of labeled TERE1 and apoE proteins can be compared to labeled luciferase, the kits positive control, and TBL2 products. **B**: Affinity purification of in vitro translated T7-apoE (**lane 1**) labeled with ^{35}S-methionine/cysteine with bound 6XHis-TERE1 (labeled with ^{35}S-methionine/cysteine, **lane 2**).

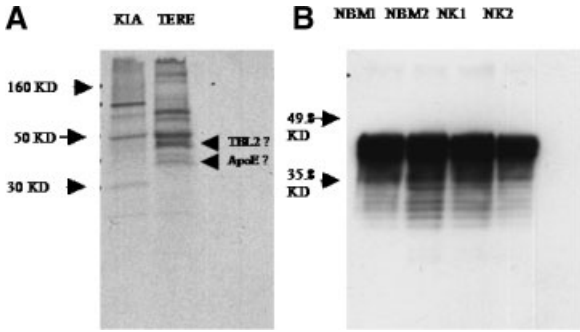

Fig. 2. Association of various sized proteins with TERE1. **A**: Flurogram of an SDS-poyacrylamide gel containing proteins immunoprecipitated with a polyclonal antibody against the KIAA1096 or the TERE1 from lysates of ^{35}S methionine-labeled 1376 TCC cells. **B**: Western blot of an SDS–PAGE containing proteins immunoprecipitation with the monoclonal antibody to apoE and from normal bladder and kidney and detect with the polyclonal TERE1 antibody.

Human 293 cells were then transiently transfected with two different apoE expression vectors. High levels of expression of apoE expression were detected using a specific monoclonal antibody (Santa Cruz Biotechnology) (Fig. 4A). The expression of the HisC- truncated apoE was not detected in transfected 293 cells by this antibody due to the apparent loss of the epitope between AA124-191 (Fig. 4A). We were, however, able to detect the 6XHis tagged truncated apoE protein with the X-press antibody (Fig. 4B).

As previous studies have indicated, apoE expression can decrease MAP kinase phosphorylation in rat fibroblasts [Ho et al., 2001]. MAP kinase phosphorylation was examined using a specific monoclonal antibody in control and apoE transfected human 293 cells after 24 and 48 h (Fig. 5C) compared to MAP kinase expression (Fig. 5B). At 48 h, there was a decrease in MAP kinase phosphorylation in apoE transfected cells compared to either control cells or ApoE transfected cells after 24 h. The change in phosphorylation of MAP kinase was not due to the concentration of apoE protein given the similar levels of apoE protein after 48 h compared to the apoE concentration at 24 h (Fig. 5A). Decreased levels of phosphorylated MAP kinase were also observed in cells transfected with the 6XHis-truncated ApoE.

The effect of extracellular apoE was examined on the UMUC-3 TCC cell line using recombinant apoE secreted into media by apoE transfected 293 cells or recombinant ApoE. Again, we examined MAP kinase (p42/44) phosphorylation over a short time period (1, 3, and 6 h). A significant decrease in MAP kinase phosphorylation was observed at 3 h after ApoE treatment compared to serum treated cells (data not shown).

DISCUSSION

In this study using the Bacterial two hybrid system, we found a clone containing the C-terminal portion of the preapolipoprotein E sequence starting from amino acid 124. This interaction with the TERE1 construct was first isolated from two multiple antibiotic resistant clones and after secondary isolation using X-gal/IPTG as a substrate. The bacterial two hybrid system, as compared to conventional yeast two hybrid systems, allows for the screening of larger libraries. In addition, the use of *E. coli* makes this technique much faster than yeast systems. This interaction between the TERE1and apoE proteins was confirmed by immunoprecipitation with a TERE1 specific antibody and affinity chromatography.

We have previously shown by IHC in human prostate that TERE1 localizes largely to the apical cytoplasm of epithelial cells [McGarvey et al., 2003]. The reported intracellular distribution of apoE appears to be similar to the TERE1 expression pattern [Ho et al., 2000]. Exogenous apoE expression in macrophages has been reported to be largely endosomal while

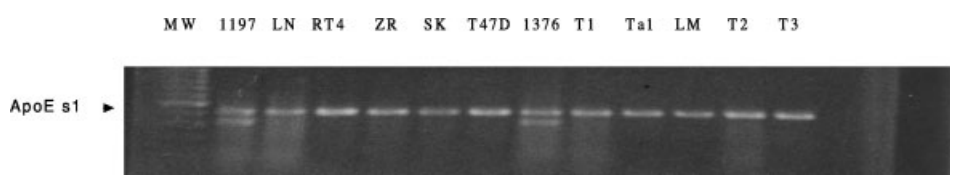

Fig. 3. cDNA derived from various tumor cell lines (1197 TCC cell line; LNCaP prostate carcinoma cells; RT4 TCC cell line; ZR, SK, and T47D breast carcinoma cell lines; and 1376 TCC cell line) and from frozen human bladder tissue [including T1 stage, Ta1, lymph node metastasis (LM), T2 stage, and T3 stage bladder carcinomas] was used as a PCR template using apoE specific primers. ApoE transcript was observed in all samples including normal bladder mucosa. No significant difference in steady state expression was observed between the tumor cell lines, normal bladder samples, or any of bladder tumor specimens.

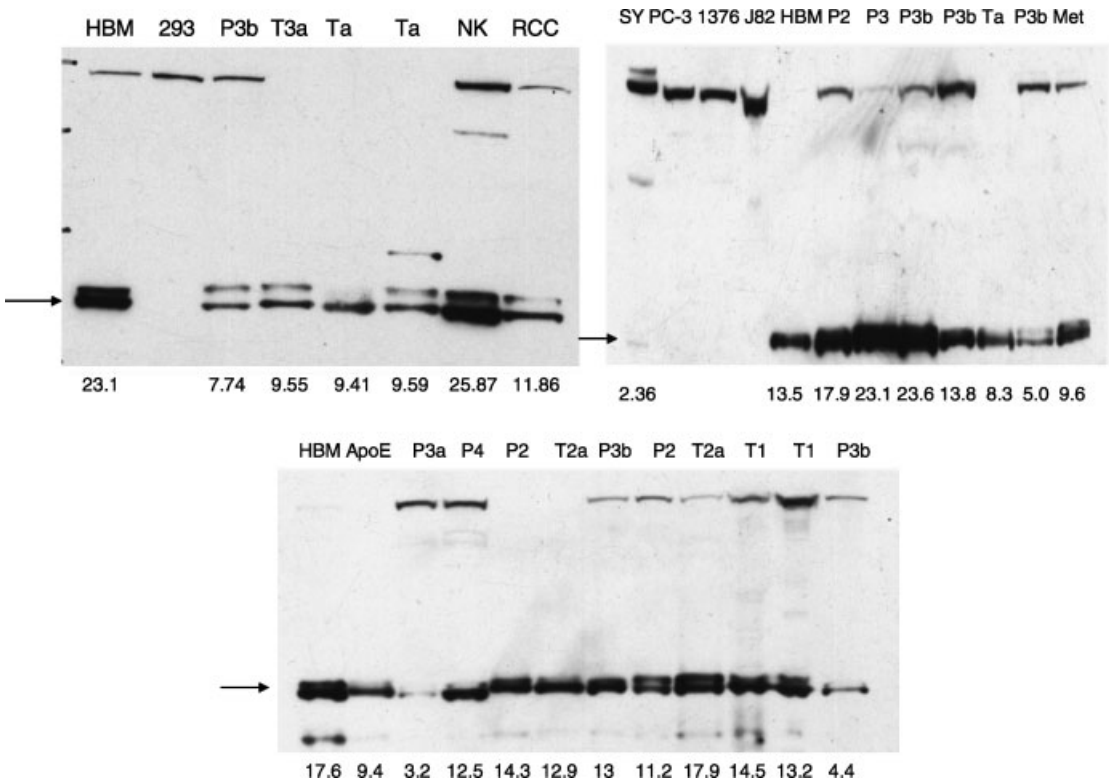

Fig. 4. ApoE Western blotting. Immunoblotting was then performed using an apoE specific antibody on total protein derived from TCC tumor cells, the 293 human embryonic kidney cell line, normal human bladder and kidney as well as various bladder and renal cell carcinoma tumor tissue samples. Protein from three different normal bladder tumor samples were also examined. The arrow points to the 34 kD apoE specific band. Overall, we found that there was an apparent decrease in ApoE protein levels in the 21 TCC and 1 RCC samples as compared to 3 samples of normal human bladder mucosa and normal human kidney (NK). Volumetric analyses were performed on scanned images, and the values are added below each lane. Pathologic stage for each sample is above each lane and is based on the TNM system for bladder cancer. A Ta tumor is a noninvasive papillary carcinoma. A T1 tumor is a tumor that invaded the sub-epithelial connective tissue. A T2 tumor has invaded the bladder muscle (T2a, superficial muscle). A T3 tumor has invaded perivesical tissue (pT3a–microscopic invasion, pT3b–macroscopic).

endogenous apoE localizes within saccular distensions of all saccules of stacked Golgi cisternae [Ho et al., 2000]. While apoE localizes to the cytoplasm in apoE transfected rat fibroblasts, there is not a complete overlap with the Golgi staining pattern [Ho et al., 2001].

Using Western blotting, we have found that normal bladder mucosa and bladder tumor specimens contain the apoE protein. Using RT/PCR with apoE specific primers, we found that apoE is also expressed by normal and malignant urothelium including various TCC cell lines. This indicates that a least a portion of apoE protein detected by Western blotting is due to expression by normal urothelial cells in the bladder mucosa or tumor cells. Multiple extrahepatic tissues such as the kidney, stomach, intestine, lung, spleen, testes, ovary, and brain have also been found to synthesize apoE [Law et al., 1997]. In the kidney, ApoE has been found to regulate mesangial cell proliferation [Chen et al., 2001]. From this data, one hypothesis that might be constructed is that apoE expression may partially regulate proliferation in the bladder. Previously, we have found transient or stable expression of TERE1 in TCC cells decreased proliferation [McGarvey et al., 2001]. A number of downstream early genes were up-regulated while late G1 genes appeared to be down-regulated in a micro array analysis of a TERE1 over expressing TCC cell line (unpublished data). In this microarray analysis, TERE1 transduced J82 cells were also found to have a 2.5 fold increase in the expression of apoE as compared to control transduced cells. It is interesting to speculate that the interaction between apoE and TERE1 may enhance cell cycle arrest.

The Western blot data in this study indicates a 50% reduction in the apoE protein in 7 of 14

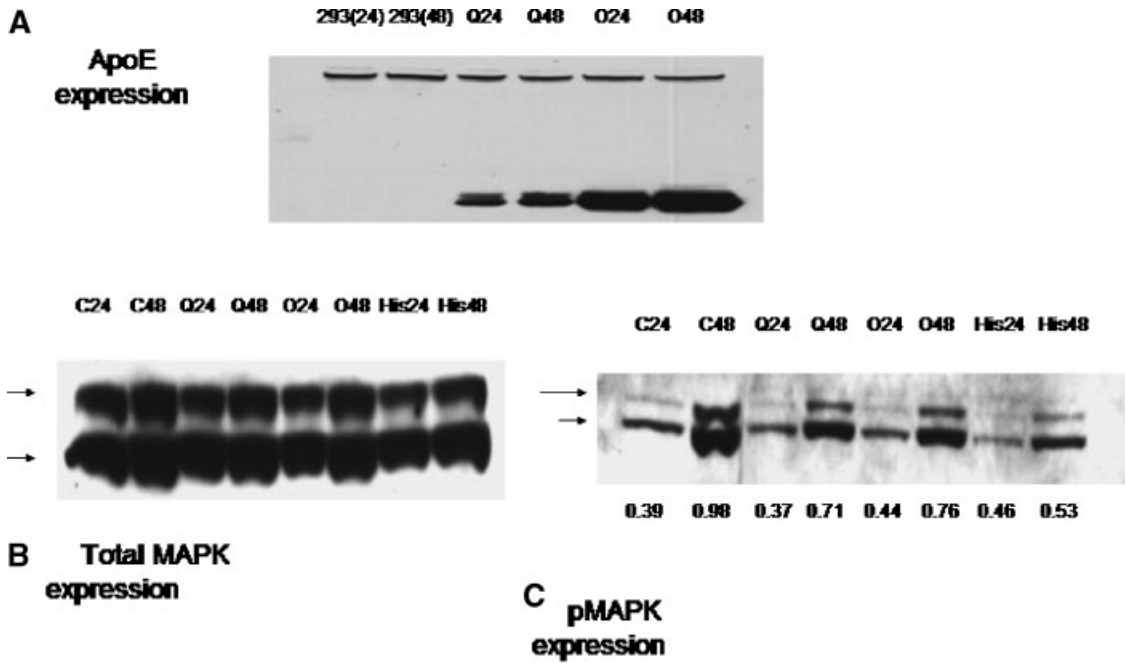

Fig. 5. ApoE/MAP kinase levels Western blots in 293 cells. ApoE expression was examined in control, ApoE transfected, and truncated 6XHisApoE cells (Fig. 5A). Total MAP kinase was examined using a specific monoclonal antibody in control and apoE transfected human 293 cells after 24 and 48 h (Fig. 5B) compared to the levels of phosphorylation MAP kinase expression (Fig. 5C). The ratio of the comparision of the volumetric analysis between total MAP kinase levels versus phosphorylated MAP kinase levels is below each respective lane. At 48 h, there was a decrease in MAP kinase phosphorylation in apoE transfected cells compared to either control cells or ApoE transfected cells after 24 h. A similar decrease was observed in 293 cells transfected with the truncated apoE construct.

invasive and 5 of 10 superficial TCC specimens. However, recent reports have linked either apoE transcript or apoE protein overexpression as determined by immunohistochemistry (IHC) to ovarian and prostate cancer [Hough et al., 2000; Venanzoni et al., 2003]. In addition, increased expression of the apoE transcript was observed in various prostate tumors that had low expression of FAS (fatty acid synthase) transcript but high levels of FAS protein [Rossi et al., 2003].

MAP kinase phosphorylation was significantly decreased in apoE or 6XHis-truncated ApoE transfected cells compared to either control transfected cells or ApoE transfected cells after 24 h. Moreover, there was no difference in total p42/p44 MAP kinase levels in control or transfected 293 cells. These results are in agreement with the effect of apoE expression on rat F111 embryonic fibroblasts as described by Ho et al. [2001]. These investigators found that endogenous apoE inhibits cellular proliferation/DNA synthesis in the presence of serum, but stimulates DNA synthesis in the absence of serum [Ho et al., 2001]. This inhibition of proliferation has been reported to be mediated through the MAPK pathway but not through the induction of c-fos [Ho et al., 2001]. On the other hand, various laboratories have shown that exogenous apoE inhibits cellular proliferation [Vogel et al., 1994; Ishigami et al., 1998]. This data does not exclude an exogenous receptor mediated response to apoE. Without inhibiting the lipoprotein receptor it is difficult to determine whether the effect on the phosphorylation of MAP kinase is due to endogenous ApoE or receptor-mediated exogenous ApoE produced and secreted by the transfected 293 cells. An increase in ApoE transcript in various tumor cell lines upon serum induced growth arrest was reported [Do Carmo et al., 2002]. In vitro, the MAP kinase pathway has been shown to be significant factor in controlling proliferation in normal urothelial cells, but seem to be less important in various TCC cell lines [Swiatkowski et al., 2003]. Our own analysis of TCC specimens compared to normal bladder mucosa indicates, however that there is an increase in phosphorylation of p44/p42 in the tumor samples (data not shown).

The effect of extracellular apoE was examined on various TCC cell lines using recombinant

apoE secreted into media by apoE transfected 293 cells. Again, we examined MAP kinase (p42/44) phosphorylation over a short time period (1, 3, and 6 h). We found a significant decrease in MAPK activation (phosphorylation) in serum and apoE treated UMUC-3 cells compared to serum stimulated UMUC-3 cells. A similar decrease in MAP kinase phosphorylation was found in non-transfected rat fibroblasts [Ho et al., 2001]. Therefore, a receptor based apoE pathway is present in bladder tumor cells, however the effect of induced endogenous apoE expression on growth-related pathways in TCC cells is still under investigation.

In our screening for TERE1 interacting clones, we did not isolate the other known apoE interacting protein, the amyloid precursor protein (APP) even though APP seems to be ubiquitously expressed. The APP protein has been shown to interact with intracellular apoE protein within the first 1–191 amino acids of apoE [Hass et al., 1998]. Therefore, even though there is a partial overlap in interacting domains, we had not isolated APP in the any of the positive bacterial clones. A further examination of apoE/TERE1 truncations will be crucial in defining the TERE1 interaction site or sites. The critical question of functional consequences of the interaction between TERE1 and apoE remains to be elucidated. Interestingly, there is an apoE chaperon-like function that has been proposed in the interaction with amyloid protein implying that TERE1 does not have a role in regulating this chaperon function [Hass et al., 1998].

In summary, we have documented an apparent interaction between TERE1 and ApoE. Both proteins have a role in controlling cellular proliferation. Defined knockouts and functional assays may yield further clues to determining if the interaction between TERE1 and ApoE has an important role in growth-related signal transduction pathway(s).

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