SH3RF2 functions as an oncogene by mediating PKA4 protein stability

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SH3RF (SH3-domain-containing RING finger protein) family members, SH3RF1-3, are multidomain scaffold proteins involved in promoting cell survival and apoptosis. In this report, we show that SH3RF2 is an oncogene product that is overexpressed in human cancers and regulates p21-activated kinase 4 (PAK4) protein stability. Immunohistochemical analysis of 159 colon cancer tissues showed that SH3RF2 expression levels are frequently elevated in cancer tissues and significantly correlate with poor prognostic indicators, including increased invasion, early recurrence and poor survival rates. We also demonstrated that PAK4 protein is degraded by the ubiquitin–proteasome system and that SH3RF2 inhibits PAK4 ubiquitination via physical interaction-mediated steric hindrance, which results in the upregulation of PAK4 protein. Moreover, ablation of SH3RF2 expression attenuates TRADD (TNF-α-associated death domain) recruitment to tumor necrosis factor-α (TNF-α) receptor 1 and hinders downstream signals, thereby inhibiting NF-κB (nuclear factor-kappaB) activity and enhancing caspase-8 activity, in the context of TNF-α treatment. Notably, ectopic expression of SH3RF2 effectively prevents apoptosis in cancer cells and enhances cell migration, colony formation and tumor growth in vivo. Taken together, our results suggest that SH3RF2 is an oncogene that may be a definitive regulator of PAK4. Therefore, SH3RF2 may represent an effective therapeutic target for cancer treatment.

Introduction

The tumor microenvironment is an important determinant of tumor growth and survival. Tumor cells release a variety of cytokines and chemokines, thereby recruiting and promoting infiltration by immune cells such as monocytes and macrophages, which also release various cytokines, including tumor necrosis factor-α (TNF-α) (1,2). TNF-α is a multifunctional cytokine involved in diverse cellular events, such as cell proliferation, inflammation, differentiation and apoptosis (3,4). TNF-α transduces signals through two distinct cell surface receptors, TNF receptor 1 (TNFR1) and TNFR2 (5). TNFR1 activation can have two different end results. The binding of TNF-α to TNFR1 leads to the initial receptor complex of TNFR1 (complex I) associated with adaptor protein TNFR-associated death domain (TRADD), which recruits additional adaptor proteins receptor-interacting protein (RIP), TNFR-associated factor 2 (TRAF2). This complex triggers signals leading to the activation of nuclear factor-kappaB (NF-κB) and c-Jun N-terminal kinase (JNK). In the second step, both TNFR1 and the adaptor proteins can undergo conformational changes that lead to the internalization of the signaling complex and to changes in the adaptor proteins that bind to the receptor. This new signaling complex (complex II) differs from complex I in that TRADD, caspase-8 and FADD also interact with TNFR1. This secondary complex leads to the activation of caspase-8 and thus to the triggering of the apoptotic process (6,7). However, the use of TNF-α as a treatment for cancer is hindered by the development of resistance to TNF-α treatment. Notably, ectopic expression of two transcription factors, activating protein-1 (AP-1) and NF-κB, may account for TNF-α resistance in cancer cells and the subsequent promotion of tumor development (8,9). However, the detailed molecular mechanisms underlying the abnormal activation of these transcription factors and the acquisition of TNF-α resistance have not been clearly elucidated.

SH3RF1 (SH3-domain-containing RING finger protein 1: POSH, plenty of SH3s), a member of three multidomain scaffold proteins containing a RING (really interesting new gene) domain and multiple SH3 (Src-homology 3) domains, plays an important role in the TNF-α signaling pathway. Drosophila POSH promotes survival against Eiger-induced cell death by possibly acting downstream of dTAB2 and upstream of dTAK1 in the Eiger-Basket (corresponding to mammalian TNF-JNK) signaling pathway (10). Additionally, human POSH is highly expressed in human rheumatoid arthritis synovial fibroblasts in which it functions to increase cell survival (11), thereby suggesting the involvement of POSH in inflammation-related diseases, although there is no direct evidence for a role in TNF-α signaling. Moreover, human POSH stimulates the phosphorylation of c-Jun and activity of the AP-1 transcription factor, which is an important role in cell proliferation and in tumor promotion and progression in various type of cancer (12). SH3RF2 (also referred to as POSHER; NCBI Reference Sequence: NM_152550.3), a homologue of SH3RF1, contains three SH3 domains as well as a RING domain (13). So far, the function of SH3RF2 has remained entirely unexplored, excepting promotion of the survival of PC12 neuronal cells by deregulating POSH protein expression (14). These observations raise interesting questions regarding the mechanism by which SH3RF family members promote survival in pathologic cells.

p21-activated kinases (PAKs) are a family of serine/threonine kinases initially identified as targets of the Rho GTPases, Cdc42 and Rac (15). Although they were originally identified as proteins that regulate cell morphology, cytoskeletal reorganization and motility, PAKs have since been shown to play an important role in the regulation of cell survival, apoptosis, migration and invasion (16). Recent studies demonstrating that PAK4 overexpression promotes in vivo tumorigenesis and anchorage-independent growth in cultured cells have established PAK4 as an important oncogene that confers apoptosis resistance in cancer cells in response to TNF-α treatment, UV irradiation or serum starvation (17). In response to TNF-α, PAK4 facilitates TRADD recruitment to TNFR1 to form signal complex I, thus...
activating cell survival pathways involving the activation of NF-xB and extracellular signal-regulated kinase; it also inhibits caspase-8 activation (18).

In this study, we demonstrate that SH3RF2 is a novel PAK4-interacting protein significantly overexpressed in colon cancers and that PAK4 undergoes ubiquitin (Ub)–proteasome system-mediated degradation, which is inhibited by SH3RF2. Our results indicate that the functional interaction between SH3RF2 and PAK4 provides a mechanistic basis by which SH3RF2 promotes tumorigenesis in vitro and in vivo, thereby suggesting it an interesting oncogenic target for therapeutic intervention.

Materials and methods

Cells and reagents

The SW620, SW480, HT29, Hep3B, HepG2, SK-Hep1, PLC/PRF/5, HEK293T, HeLa cell lines and IMR90 cell lines were obtained from ATCC (American Type Culture Collection), and Hub-7 cells were obtained from the JCRB Cell Bank (Japanese Collection of Research Bioresources Cell Bank). HCT116 (p53−/−) cells were kindly provided by Dr C.W.Lee (Sungkyunkwan University, Korea) (19). The indicated antibodies against the following proteins were used: SH3RF2 (H00153769-M01; Abnova and HPA035903; Sigma), caspase-8 (#9746; Cell Signaling), TRADD (610572; BD Biosciences), GAPDH (MAB374; Millipore), cleavage-site-specific PARP (AB3565; Millipore), Flag epitope (F3165; Sigma), HA epitope (MMS-101R; Covance), phospho-JNK (#9255; Cell Signaling), JNK2 (#9258; Cell Signaling), phospho-PAK4 (#3241; Cell Signaling), PAK4 (#3242; Cell Signaling), phospho-c-Jun (sc-16312; Santa Cruz Biotechnology), β-Tubulin (sc-5274; Santa Cruz Biotechnology), NF-κB p65 (sc-8008; Santa Cruz Biotechnology), FLIP (sc-5276; Santa Cruz Biotechnology) and TNFR1 (sc-8436; Santa Cruz Biotechnology).

Recombinant human TNF-α was purchased from R&D Systems (St. Louis, TA), cycloheximide (CHX) was obtained from Sigma, and RNAiMAX was obtained from Invitrogen (13778-150). siRNAs against SH3RF2 were purchased from Dharmacon (DH-2100302); of the oligonucleotides included in the set, siRNA5# and siRNA6# were used. siRNA plasmid DNA against human SH3RF2 was obtained from Sigma (TRCN0000014793; SH3RF2MISSION shRNA); the pLKO.1-puro vector was used as a control.

Patient samples

Colon cancer samples were obtained from patients undergoing routine surgery for colon cancer at the Department of Surgery, Inje University Paik Hospital (Seoul, Korea) between November 1998 and August 2004. The colon cancer tissues were isolated from the primary cancers of 159 colon cancer patients, and when possible, from the lymph nodes of 66 metastatic colon cancer patients. The clinical status of each patient was classified according to the pathologic tumor grade, tumor size, lymph node status and Duke’s colon cancer stage. Experiments involving human tissue specimens were reviewed and approved by the Internal Review Board (IRB No.: IIT-2011–197).

Reverse transcription and quantitative real-time polymerase chain reaction

Total RNA was extracted from cells and tissues using the TRizol reagent (Invitrogen), and 4 µg each RNA sample was reverse transcribed using reverse transcriptase (Thermo Scientific) according to the manufacturer’s guidelines. The reverse transcription–polymerase chain reaction (RT–PCR) experiments were performed according to standard protocols, with the following optimized PCR conditions: 1 cycle at 95°C for 30 s; 18–32 (for RT–PCR) or 45 (for quantitative real-time PCR) cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s and a final extension cycle at 72°C for 10 min. Quantitative real-time RT–PCR was performed in a CFX96 Real-Time PCR Detection System (Bio-Rad). The reverse-transcribed product was amplified in a 20 µl reaction with FastStart Universal SYBR Green Master (Roxy) (Roche). PCR was amplified using the following primers: SH3RF2 (forward 5′-AAGAGGGAAGAGGCTCAGG-3′ and reverse 5′-TGGAGCGGAAGGACCATTG-3′); PAK4 (forward 5′-GAGCTGCTCTTCCAAGAGGT-3′ and reverse 5′-CTCTGGATCTAGTGCAGT-3′); β-actin (forward 5′-CTGGAGAAGGAGCTACGAGTC-3′ and reverse 5′-GCTGAGACGGTATCTGCG-3′). Relative gene expression levels were calculated as 2−ΔΔCt with β-actin used for normalization, where Ct was defined as the threshold cycle of PCR at which amplified product was first detected.

Tissue microarrays and immunohistochemical analysis

Tissue microarray (TMA) analyses were performed following a previously reported protocol with slight modifications (20). The tissue array blocks contained 159 colon cancer tissues and 20 non-neoplastic colonic mucosal epithelial tissues. Sections of the tissue array blocks (4 µm thick) were analyzed immunohistochemically. Following deparaffinization and antigen retrieval, the sections were labeled with antibodies against the avidin–biotin complex method with 3,3′-diaminobenzidine as a chromogen. Normal saline was used as a substitute for the primary Ab in the negative control reactions. SH3RF2 expression was scored as positive if ≥20% of the cells showed moderate to strong staining, weak if either the cytoplasmic or membranous staining was <20% and negative if neither cytoplasmic nor membranous staining was observed. For statistical analysis of the TMA data, categorical variables were compared using chi-square test and analysis of variance (ANOVA). The Statistical Package for the Social Sciences (version 11.01) statistical software version 11.0.1. Survival rates were compared using Kaplan–Meier plots for univariate analysis using the same software. A P value <0.05 was regarded as significant.

Capillary reverse-phase liquid chromatography–tandem mass spectrometry analysis

Enzymatic digestion and liquid chromatography–tandem mass spectrometry (LC–MS/MS) analyses of immunoprecipitated protein samples were performed as described previously (21). Briefly, proteins eluted from Ab beads were digested with sequencing-grade modified trypsin (Promega) for 15 h at 37°C at an enzyme-to-substrate ratio of 1:25 in 100 mM Tris–HCl (pH 8.0) buffer containing 5 mM CaCl2. Fully tryptic digested proteins were loaded onto fused silica capillary columns (100 µm internal diameter, 360 µm outer diameter), and the eluted peptides were directly electrosprayed into an LTQ Ion Trap mass spectrometer (Thermo Finnigan) by applying 2.3 kV of DC voltage. MS/MS spectra were compared with the human IPI protein database (version 3.37) using TurboSequest and SEQUEST Cluster Systems (14 nodes); DTASelect was used to filter the search results.

Soft agar assay

Six-well plates were filled with 2 ml of 0.6% Bacto agar in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, antibiotics and glutamine (10×). Twenty cells in 1 ml of serum-free medium were seeded on the surface of the agar, and the same medium was seeded onto the plates at 5000 or 10 000 cells per well. Each cell line was tested in duplicate. After 2–3 weeks, the colonies were visualized using an inverted light microscope. Digital images of each well were also taken.

Transwell migration assays

Transwell migration assays were performed in 24-well Transwell chamber (Corning, NY) fitted with a polycarbonate membrane (8 µm pore size). Cells were washed twice with serum-free medium, resuspended in 1 ml of 2 × 105 cells per well serum-free medium and added to the upper chamber. The lower chamber was filled with Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. After 24 h, migrated cells were fixed for 20 min with methanol and stained with 0.25% crystal violet, 10% formaldehyde and 80% methanol for 6 h, and then washed with ddH2O to remove non-adherent cells. Ten random fields were captured under ×100 magnification for each membrane, and migrated cells were counted.

Apo-ONE homogeneous caspase-3/7 assay

Caspase-3 and caspase-7 activity were determined in a black 96-well plate using Apo-ONE™ Homogeneous Caspase-3/7 Reagent (G7792; Promega) following the manufacturer’s protocol. The fluorescence of each well was measured at excitation and emission wavelengths of 499 and 521 nm, respectively.

Western blot and immunoprecipitation

Cells were lysed in radioimmunoprecipitation assay buffer (RIPA) buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40 and 1 mM ethylenediaminetetraacetic acid) containing protease inhibitor cocktail (008387001; Roche) and protease inhibitor cocktail (1169749801; Roche), incubated on ice for 30 min and then collected by centrifugation at 10 000g for 10 min. The soluble fractions were collected as cell lysates and used for immunoprecipitation assays. For direct immunoprecipitation of each protein, a target protein, a target protein (IgG (1 µg of IgG/500 µg of lysate protein) was added to the cell lysate and incubated for 6 h or overnight at 4°C with rotation (inversion). A total of 30 µl of protein G agarose slurry (sc-202; Santa Cruz Biotechnology) was added, and the reaction mix was incubated with rotation for another 2 h at 4°C. For immunoprecipitation of Flag- or Myc-epitope-tagged proteins, agarose beads preloaded with the antibodies for the specific epitope tag [anti-Flag
M2 affinity gel for Flag epitope (A2220; Sigma), Red Anti-ε-Myc affinity gel for Myc epitope (E6654; Sigma]) were added directly to the cell lysates expressing the tagged protein(s) and then incubated for 6 h or overnight at 4°C with rotation. All immune complexes were collected by centrifugation and washed three times with RIPA buffer. Endogenous TNFR protein was immunoprecipitated using PureProteome Protein G Magnetic beads (LSKMAGG10; Millipore) according to the manufacturer’s protocol. The precipitated proteins were subjected to western blot analysis. For immunoblotting, membranes were incubated with the primary antibody (1:1000) for overnight, followed by 1 h incubation with 1:4000 dilution of horseradish peroxidase-linked secondary antibody, membranes with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or β-actin were probed with corresponding antibody to assure equal to loading. Finally, the immunoreactive proteins were detected by enhanced chemiluminescence assay with horseradish peroxidase (Pierce).

Glutathione-S-transferase pull-down assay

Direct protein–protein interactions were identified using purified proteins and glutathione-5-transferase (GST) pull-down assays. The GST-SH3RF2 expression vectors were constructed by inserting the amplified full-length SH3RF2 or SH3RF2 fragment cDNAs into the EcoRI and SalI sites of the pGEX-4T-2 vector (28-9545-50; Amersham pharmacia biotech). The His-PAK4 expression vector was constructed by inserting the amplified PAK4 cDNA into the EcoRI and Xhol sites of the pET28a vector (69684-3; Novagen). GST fusion construct and His fusion construct were produced in Escherichia coli BL21(DE3) pLysS, and the fusions proteins were induced with 0.5–1 mM isopropyl-D-thiogalactopyranoside for 3–6 h.

For binding assays, the GST, GST fusion proteins and His-PAK4 proteins were mixed and rotated for 6 h at 4°C. After 30 µl glutathione-Sepharose beads were added, the mixture was further incubated for 4 h at 4°C with rotation.

The protein complex was pulled down via centrifugation at 1200 r.p.m. for 1 min and washed four times with RIPA buffer. Bound proteins were then eluted with 2x sodium dodecyl sulfate loading buffer. The GST fusion protein complex was detected via Coomassie Blue staining or western blotting.

Ubiquitination assay

For the PAK4 ubiquitination assays, plasmids encoding SH3RF2 or SH3RF2∆RING were individually cotransfected into HEK 293T cells at 50–60% confluence with the vectors for PAK4-Flag and Ub-HA. Forty-eight hours after transfection, the cells were harvested, washed with phosphate-buffered saline, pelleted and lysed in RIPA buffer. PAK4-Flag was precipitated from cell lysates by affinity purification using anti-Flag M2 affinity gel. Precipitated PAK4 proteins were resolved via sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analyzed via western blotting with an anti-HA Ab.

Tumor growth in nude mice

Male BALB/c nude mice (4–6 weeks old; 20 ± 2 g) were purchased from Japan SLC. Mice were kept under specific pathogen-free conditions and maintained on a standard diet. Cells (2 x 10^6) were injected subcutaneously into the flanks of nude mice using a 26-gauge needle. Each cell line was tested in five different animals.

Results

Significant overexpression of SH3RF2 in cancers

In an analysis of SH3RF2 family gene expression using recently published microarray-based transcriptome data on 66 colon cancer tissues (22), we found that the expression of the SH3RF2 gene was significantly higher in cancer tissues compared with non-cancerous tissues (n = 9) (Figure 1A). SH3RF2 protein expression was also upregulated in various cancer cell lines, including several human hepatocellular carcinoma cell lines, colon cancer cell lines, cervical cancer cell line and embryonic kidney cell lines (HEK293T cell), compared with the human diploid lung fibroblast strain IMR90 (Figure 1B).

To evaluate the clinical significance of SH3RF2 expression, we performed immunohistochemical analyses using TMAs consisting of 159 colon cancer tissues that were surgically resected at Inje University Seoul Paik Hospital. In a primary analysis with non-neoplastic colonic mucosal epithelial tissues, we found that SH3RF2 protein was weakly expressed (staining intensity, 1+) exclusively in the nucleus of growing crypt base cells (Figure 1C). Of the 159 colon cancers examined, 93 (58.5%) clearly exhibited SH3RF2 overexpression with much stronger intensity (2+) and a distinct, mostly nuclear staining pattern.

In the remaining 66 (41.5%) colon cancers, the SH3RF2 levels were much lower or undetectable. SH3RF2 expression was more frequent and stronger (P < 0.001) in the infiltrating peripheral region (88 cases; 55.3%) than in the central region (40 cases; 25.2%) of the tumor mass, thereby suggesting the possible infiltration of SH3RF2-positive colon cancer cells into adjacent non-tumor tissues.

The clinicopathologic significance of SH3RF2 upregulation in colon cancer was evaluated by correlating the expression pattern with clinical parameters. Colon cancers displaying SH3RF2 overexpression were associated with high TNM (T NM classification of malignant tumors) stage, high lymphatic invasion, high venous invasion and early recurrence (P < 0.01) (Table 1). Additionally, we found that SH3RF2 expression in tumor masses displayed a significant positive relationship with clinical indicators of poor outcome. The SH3RF2 expression-positive group demonstrated both worse overall and disease-free survival than the SH3RF2 expression-negative group according to Kaplan–Meier analyses (Figure 1D). These data suggest that SH3RF2, whose overexpression was correlated with aggressive tumor behaviors accompanying tumor metastasis and survival, could be used as a potential new prognostic marker for the aggressiveness of colon cancer. Furthermore, these results imply that SH3RF2 may play an important role in tumor metastasis and survival.

SH3RF2 physically interacts with PAK4 protein

To elucidate the cellular function of SH3RF2, we aimed to identify its interacting proteins with known functions and performed immunoprecipitation analysis combined with LC–MS/MS. For these experiments, lysates of SK-HeP1 cells transduced with pcDNA/SH3RF2-Myc vectors (SK-HeP1/SH3RF2-Myc#1 or SK-HeP1/SH3RF2-Myc#3) or the pcDNA-Myc vector (SK-HeP1/control) were immunoprecipitated using an anti-Myc antibody (Ab), and the immunoprecipitation samples were then subjected to LC–MS/MS spectrum analysis. Proteins corresponding to 14 genes were identified as common to the immunoprecipitation samples derived from two independent SH3RF2-Myc-expressing cell types but absent from the control cells (Table II; Supplementary Table 1, available at Carcinogenesis Online).

Interestingly, the list included PK4, which is an important oncogene that causes oncogenic transformation in cells and mouse models (23, 24).

To validate whether SH3RF2 interacts with PK4 protein, we used HeLa cells transiently cotransfected with pcDNA/PAK4-Flag and either pcDNA/SH3RF2-Myc or empty pcDNA vector (control). Cell lysates were subjected to immunoprecipitation and western blot analysis using anti-Myc and anti-Flag antibodies, respectively. PK4 co-precipitated with SH3RF2, thereby verifying that PK4 protein interacts with SH3RF2 protein in intact cells (Figure 2A). These results were confirmed in the complementary experiment using anti-Flag affinity gel to immunoprecipitate PK4 from cell lysates and an anti-SH3RF2 Ab to probe for endogenous SH3RF2 protein (Figure 2B).

Furthermore, we identified the interaction between endogenous SH3RF2 and PK4 proteins from parent HT29 cell lysates by using anti-PK4 Ab to immunoprecipitate endogenous PK4 and anti-SH3RF2 Ab to probe for endogenous SH3RF2 protein (Figure 2C).

To determine whether this interaction was direct, we also performed GST pull-down assays using GST-SH3RF2 and His-PK4 proteins expressed in and purified from E.coli. The results clearly showed that PK4 directly interacts with SH3RF2 (Figure 2D), especially with the C-terminal portion of the PK4 protein (Supplementary Figure 1A and B, available at Carcinogenesis Online). Using various GST-fused SH3RF2 fragment mutants prepared to determine the region of SH3RF2 that binds to the PK4 protein (Figure 2E), we found that the third SH3 domain of SH3RF2 protein (370–459) is the specific PK4-binding region (Figure 2F).

PAK4 protein undergoes Ub–proteasome-mediated degradation, which is inhibited by direct binding with SH3RF2

Next, we sought to determine whether there is a causal relationship between SH3RF2 and PK4 protein expression. Interestingly, we found
SH3RF2 regulates PAK4 protein stability

Fig. 1. SH3RF2 is frequently overexpressed in human cancer cell lines and clinical cancer tissues and is associated with survival and recurrence of colon cancer patients. (A) The expression pattern of the SH3RF2 gene was analyzed using Illumina chip data of 66 colon cancer tissues and the corresponding 9 non-tumor colon tissues. The Student’s t-test was used to test differences in mRNA expression between colon cancer tissues and adjacent normal tissues. P values were expressed as the mean ± SD of the mean. (B) SH3RF2 proteins were analyzed in multiple cancer cell lines compared with a normal cell line, IMR90, via western blot analysis using anti-SH3RF2 Ab. S3R2 represents SH3RF2. β-Actin was used as a loading control. (C) A representative immunohistochemical staining for SH3RF2 in a colon cancer tissues and the corresponding non-tumorous colon tissues. CC represents colon cancer tissues. (D) Kaplan–Meier analysis of the overall and disease-free survival of colon cancer patients based on the immunohistochemical staining results. For survival analysis, the patients were subdivided into positive or negative according to the extent of SH3RF2 protein expression (see Materials and methods).
that PAK4 protein levels were significantly increased in the SH3RF2-overexpressing PLC/PRF/5 (S3R2#3) and SK-Hep1 (S3R2#3) cell lines without affecting PAK4 mRNA levels ([Figure 3A]; Supplementary Figure 2A, available at Carcinogenesis Online). Consistent with the SH3RF2-dependent upregulation of PAK4 protein, we found that PAK4 was downregulated in stably transfected SH3RF2 knockdown cell lines (shS3R2#1, shS3R2#3; [Figure 3B]; Supplementary Figure 2B, available at Carcinogenesis Online). Because SH3RF2 binds with PAK4 and positively regulates PAK4 protein expression without affecting PAK4 mRNA levels, we hypothesized that SH3RF2 might regulate PAK4 protein stability possibly by modulating Ub-dependent proteasomal degradation. To test this hypothesis, we first examined the effect of the proteasome inhibitor MG132 on cellular PAK4 protein levels. MG132 treatment led to a substantial increase in endogenous PAK4 protein levels in control Huh-7 (shCon.) cells as well as in Huh-7/shSH3RF2#5 cells, in which SH3RF2 has been stably knocked down via shRNA expression ([Figure 3C]). We next performed an ubiquitination assay in cells cotransfected with PAK4-Flag and SH3RF2-expressing plasmid or control vectors and found that PAK4 protein was heavily ubiquitinated in the MG132-treated sample with a characteristic ladder pattern indicative of polyubiquitination ([Figure 3D], lane 4). Additionally, the ubiquitination of PAK4 protein was dramatically inhibited in proportion to the levels of SH3RF2 protein expression ([Figure 3E]).

Meanwhile, because SH3RF2 is a ‘bona fide’ Ub ligase (see Supplementary Figure 2C, available at Carcinogenesis Online), we investigated the role of SH3RF2 Ub ligase activity in the ubiquitination and turnover of PAK4 protein. In a cell-based ubiquitination assay, we found that both the full-length SH3RF2 and a truncated SH3RF2 protein lacking the RING domain (SH3RF2∆RING) effectively inhibited PAK4 ubiquitination ([Figure 3F]). This result suggests that the physical interaction between SH3RF2 and PAK4 per se, but not the Ub ligase activity of SH3RF2, is the critical determinant of PAK4 protein expression in the cell. We further verified this result in Huh-7 and HEK293T cell lines transiently expressing SH3RF2 or SH3RF2∆RING mutant proteins (Supplementary Figure 2D, available at Carcinogenesis Online). Taken together, our findings strongly indicate that PAK4 protein is turned over via the proteasomal pathway and that SH3RF2 may protect PAK4 protein from proteasome-dependent degradation.

SH3RF2 potentiates TNF-α-induced prosurvival signals

To explore the functional significance of SH3RF2-mediated PAK4 accumulation, we analyzed TNF-α-mediated cellular signaling, because, as noted previously, PAK4 promotes TNF-α-stimulated survival pathways (18). Based on our observation that SH3RF2 directly interacts with and increases the stability of PAK4 protein, we assumed

Table I. Relationship between the SH3RF2 expression level and clinicopathological feature of colon cancer patients

<table>
<thead>
<tr>
<th>Clinical parameters</th>
<th>No. of patients</th>
<th>SH3RF2 expression</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td>71 (44.7%)</td>
<td>28</td>
</tr>
<tr>
<td>&lt;60</td>
<td>88 (55.3%)</td>
<td>38</td>
<td>50</td>
</tr>
<tr>
<td>≥60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td>89 (44.0%)</td>
<td>31</td>
</tr>
<tr>
<td>M</td>
<td>70 (56.0%)</td>
<td>35</td>
<td>58</td>
</tr>
<tr>
<td>F</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Classification</td>
<td></td>
<td>119 (74.8%)</td>
<td>53</td>
</tr>
<tr>
<td>Poly/poid/fungating</td>
<td>40 (25.2%)</td>
<td>13</td>
<td>27</td>
</tr>
<tr>
<td>Tumor site</td>
<td></td>
<td>122 (76.7%)</td>
<td>50</td>
</tr>
<tr>
<td>Left colon</td>
<td>37 (23.3%)</td>
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<td>21</td>
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<tr>
<td>Right colon</td>
<td></td>
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<td></td>
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<tr>
<td>Tumor size (cm)</td>
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<td>75 (47.2%)</td>
<td>40</td>
</tr>
<tr>
<td>&lt;5</td>
<td>84 (52.8%)</td>
<td>26</td>
<td>58</td>
</tr>
<tr>
<td>≥5</td>
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<td></td>
</tr>
<tr>
<td>T-Primary tumor</td>
<td></td>
<td>26 (16.4%)</td>
<td>22</td>
</tr>
<tr>
<td>I, II</td>
<td>133 (83.6%)</td>
<td>44</td>
<td>89</td>
</tr>
<tr>
<td>III, IV</td>
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<tr>
<td>N-Lymph nodes</td>
<td></td>
<td>73 (45.9%)</td>
<td>44</td>
</tr>
<tr>
<td>N0</td>
<td>86 (54.1%)</td>
<td>22</td>
<td>64</td>
</tr>
<tr>
<td>N1/2</td>
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<tr>
<td>M-Distant metastasis</td>
<td></td>
<td>144 (90.6%)</td>
<td>62</td>
</tr>
<tr>
<td>M0</td>
<td>15 (9.4%)</td>
<td>4</td>
<td>11</td>
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<td>M1</td>
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<td></td>
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<tr>
<td>TNM stage</td>
<td></td>
<td>73 (45.9%)</td>
<td>44</td>
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<tr>
<td>I, II</td>
<td>86 (54.1%)</td>
<td>22</td>
<td>64</td>
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<tr>
<td>III, IV</td>
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<td></td>
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<tr>
<td>Lymphatic invasion</td>
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<td>21 (13.2%)</td>
<td>17</td>
</tr>
<tr>
<td>Absent</td>
<td>138 (86.8%)</td>
<td>49</td>
<td>89</td>
</tr>
<tr>
<td>Present</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Perineural invasion</td>
<td></td>
<td>79 (49.7%)</td>
<td>43</td>
</tr>
<tr>
<td>Absent</td>
<td>80 (50.3%)</td>
<td>23</td>
<td>57</td>
</tr>
<tr>
<td>Venous invasion</td>
<td></td>
<td>121 (76.1%)</td>
<td>56</td>
</tr>
<tr>
<td>Absent</td>
<td>38 (23.9%)</td>
<td>10</td>
<td>28</td>
</tr>
<tr>
<td>Present</td>
<td></td>
<td></td>
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<tr>
<td>Differentiation</td>
<td></td>
<td>105 (66.0%)</td>
<td>53</td>
</tr>
<tr>
<td>WD/W-M</td>
<td>54 (34.0%)</td>
<td>13</td>
<td>41</td>
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<tr>
<td>M/M-P/P</td>
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<tr>
<td>Recurrence</td>
<td></td>
<td>98 (69.0%)</td>
<td>52</td>
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<tr>
<td>No</td>
<td>44 (31.0%)</td>
<td>8</td>
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that SH3RF2, similar to PK4, might also facilitate TRADD recruitment to complex I. In the immunoprecipitation assays using an anti-TNFFR1 Ab, TRADD binding to TNFR1 was reduced in SH3RF2 knockdown cells as with the effect of PK4 depletion on TRADD recruitment to complex I. In addition, other adaptor protein such as RIP-1 was reduced in SH3RF2 knockdown cells (Figure 4A). Having determined that SH3RF2 regulated the formation of TNFR1 complex, we next investigated the effects of what on TNF-α-induced signaling by constitutive knockdown of SH3RF2 expression. Our investigation of the effect of SH3RF2 on TNF-α-induced NF-κB activation showed that nuclear translocation of NF-κB p65 was significantly reduced in stable SH3RF2 knockdown cells (Figure 4B). Similarly, activation of the JNK pathway was also reduced in stable SH3RF2 knockdown cells (Figure 4C). In contrast to the NF-κB and JNK pathways, the cleaved caspase-8 and cleaved PARP (a downstream effector of caspase-8) activity (downstream caspases of caspase-8) activity (Supplementary Figure 3A) and CHX, the cells exogenously expressing SH3RF2 and is involved in cancer cell survival, we postulated that SH3RF2 ablation suppresses the tumorigenic potential of cancer cells, acts as a novel PK4-interacting protein and increases the might function as an oncogene. To assess this possibility, we examined the phenotypic characteristics of cells ectopically expressing SH3RF2. We performed anchorage-independent cell growth assays using SH3RF2 knockdown HeLa and SH3RF2-overexpressing SK-HeP1 cells. The results showed that SH3RF2 abrogation significantly reduced colony formation (Figure 5A), whereas SH3RF2 overexpression promoted colony formation (Supplementary Figure 4A, available at Carcinogenesis Online), thereby suggesting that SH3RF2 protein functions as an inducer of anchorage-independent cell growth. Additionally, blocking SH3RF2 expression clearly reduced HeLa cell migratory ability (Figure 5B), which is an important characteristic of malignant cancer cells and one of the prominent cellular phenotypes of PK4-mediated signaling.

Given that SH3RF2 knockdown effectively suppressed phenotypes associated with in vitro transformation, we investigated the effect of SH3RF2 expression in a xenograft mouse model. We established multiple stable SH3RF2 knockdown Huh-7 cell lines, in which SH3RF2 knockdown was confirmed by immunoblotting (Supplementary Figure 4B, available at Carcinogenesis Online). The growth rate of tumors formed in athymic mice inoculated with shSH3RF2#5 Huh-7 cells was significantly lower than that of tumors in the mice xenografted with control cells (Figure 5C and D). To confirm these results, we repeated the xenograft assay using shSH3RF2#7 Huh-7 cells and obtained similar results (Supplementary Figure 4C, available at Carcinogenesis Online). In contrast, in mice xenografted with PLC/PRF/5 cells stably overexpressing SH3RF2 (S3R2#3, Figure 3A), tumors developed in four of the five mice, whereas all five mice injected with control PLC/PRF/5 cells transfected with the pcDNA vector remained tumor free (Supplementary Figure 4D, available at Carcinogenesis Online). Based on these results, it is clear that SH3RF2 promotes tumor formation in athymic mice, thereby suggesting that SH3RF2 plays a role as a bona fide oncogene in cancer development.

Discussion

Because PK4 is an attractive target for anticancer therapies and its overexpression has been associated with aberrant cell survival and oncogenic transformation (25,26), understanding the mechanism of PK4 overexpression might be an important issue in cancer biology. Although genetic amplification of the PK4 gene accounts for PK4 overexpression in some tumors (27), the mechanism by which PK4 protein levels are regulated at the posttranslational level is unclear. In this report, we provide the first evidence that PK4 protein is ubiquitinated and subjected to proteasome-dependent degradation. Moreover, we showed that SH3RF2, which is upregulated in cancer cells, acts as a novel PK4-interacting protein and increases the
stability of PAK4 protein by blocking PAK4 ubiquitination via the physical interaction between SH3RF2 and PAK4 per se. These observations provide important insight into the molecular mechanisms by which PAK4 and SH3RF2 function to promote oncogenic transformation and cell survival.

A previously published work reported that PAK4 functions in TNF-α-mediated TRADD recruitment to TNFR1, which was dependent on the expression levels and kinase activity of PAK4 protein (18). However, the mechanism by which PAK4 protein facilitates the recruitment of TRADD protein has yet to be defined. Based on the study of PAK4 function in facilitating TRADD recruitment, PAK4 is likely to be found in the TNFR1 complex via its interaction with SH3RF2. Interestingly, we found the SH3RF2 protein localized to the TNFR1 complex (Supplementary Figure 5, available at Carcinogenesis Online). Therefore, PAK4 is likely to be found in the TNFR1 complex via its interaction with SH3RF2. However, we could not confirm to locate PAK4 in the TNFR1 complex, and, therefore, this issue remains an open question.

We further tested whether proapoptotic signals were also affected by FAS or TRAIL in SH3RF2 knockdown cells. As shown in the Supplementary Figure 6A and B, available at Carcinogenesis Online, both FasL and TRAIL induced apoptosis, but the extent of apoptosis was not affected by SH3RF2 knockdown. This is coincident with our results suggesting SH3RF2 function in the regulation of TRADD recruitment to TNFR1. TRADD is not a component of either FAS or TRAIL-R complex (28) and thus, SH3RF2 knockdown, we think, does not affect the apoptosis induced by FasL and TRAIL.

Moreover, we have investigated the effect of SH3RF2 knockdown on the TNF-α-dependent apoptosis in the cells treated with TNF-α only, not co-treated with CHX. We confirmed a clear induction of cleaved PARP in the SH3RF2 knockdown cells, in contrast to the no detection of cleaved PARP in the control cells with TNF-α only and cFLIP, another survival factor of TNF-α signaling, is still working (Supplementary Figure 6C and D, available at Carcinogenesis Online). Expression of FLIP is susceptible to the repressive action of CHX, a well-known inhibitor of translation. Therefore, CHX sensitizes cells to TNF-α-induced apoptosis by lowering FLIP protein level. However, TRAF2, RIP, A20 cIAP1 and cIAP2 expression levels remain unaffected upon CHX treatment (29). This help us insist that SH3RF2 might work on the upstream part, such as a regulation of TNFR1 complex I formation, in the TNF-α signaling pathway. This significant result suggested that SH3RF2 promotes TNF-α-stimulated survival pathways that is not related to the cFLIP function.

Fig. 2. SH3RF2 is a novel PAK4-interacting protein. (A) Lysates (1 mg) prepared from HEK293T cells transiently transfected with the pcDNA/PAK4-Flag and pcDNA/SH3RF2-Myc vectors were immunoprecipitated with Red Anti-c-Myc affinity gel, which was then probed for PAK4-Flag protein using an anti-Flag Ab. S3R2 represents SH3RF2. (B) HeLa cells were transiently transfected with PAK4-Flag or control vector. PAK4-Flag proteins were immunoprecipitated using anti-Flag M2 affinity gel. Immunoprecipitated samples were probed for endogenous SH3RF2 protein using an anti-SH3RF2 Ab. (C) HT29 cell lysates were immunoprecipitated with anti-PAK4 or irrelevant rabbit IgG and immunoblotted with anti-SH3RF2 Ab. (D) GST-SH3RF2 full-length proteins isolated from E. coli were mixed with His-PAK4 for the GST pull-down assay. (E) Schematic diagram of GST-SH3RF2 and the corresponding fragments used in the GST pull-down assays. The numbers indicate the amino acid position in the full-length SH3RF2 protein. (F) GST-SH3RF2 fragments were mixed with His-PAK4 and subjected to GST pull-down assay. The asterisk (*) indicates the intact size of the GST-fused protein fragments indicated at the top.
SH3RF2 regulates PAK4 protein stability

In Figure 4C, we showed that SH3RF2 knockdown alleviated JNK activation in TNF-α-treated HeLa cells. However, knockdown of rat SH3RF2 promoted stabilization of POSH protein, which is a JNK modulator, and activation of JNK signaling in neuronal PC12 cells and C6 glioma cells (14). One possible explanation of this discrepancy may be due to different working places of SH3RF2.

Our findings identified SH3RF2 as a potential oncogene that promotes cancer development by accumulating PAK4 in the cell. However, we cannot rule out the possibility that SH3RF2 may also promote tumorigenesis by modulating pathways other than TNF-α signaling. The TMA data displayed two distinct patterns of SH3RF2 protein staining in human colon cancer tissues. First, SH3RF2 protein was primarily detected in the nuclei instead of the cytosolic or membranous sites, where TNFR1 protein is localized. Recently, it was reported that PAK4 is a nucleocytoplasmic shuttling protein and interacts with and phosphorylates β-catenin, thereby promoting T-cell factor/lymphoid enhancer factor transcriptional activity (30). Additionally, PAK4 is involved in the regulation of the G1 phase and the G2/M transition of the cell cycle (31,32). These observations suggest that SH3RF2 may have additional functions in the nucleus other than regulating PAK4 protein stability. Second, SH3RF2 protein was detected more frequently and intensely in the infiltrating peripheral region of the cancer mass than in the central region. Therefore, it is tempting to speculate that SH3RF2 might induce the infiltration of colon cancer cells into adjacent non-tumorous regions, as supported by our cell migration assay results.

As SH3RF2 is a multidomain protein, it may manifest multiple oncogenic functions aside from the PAK4-mediated functions. Recent reports have shown that the increased production of cytokines, such as TNF and interleukin-6, during the inflammatory response, positively correlated with the progression of chronic viral hepatitis to hepatocellular carcinoma in humans (33,34). We speculate that SH3RF2 may play a role in important biological functions associated with other cytokines including interleukin-6. In fact, our unpublished preliminary study implies that SH3RF2 supports the antiapoptotic function of interleukin-6 in doxorubicin-induced cancer cell death (35), although the detailed mechanism remains to be determined. Further elucidation of the intracellular functions and action mechanisms of SH3RF2 as well as its interaction with tumor microenvironmental signals is necessary to fully appreciate the therapeutic value of this oncogene. In conclusion, our results suggest that SH3RF2 is a novel oncogene that promotes PAK4-mediated prosurvival function and provides a potential way for anticancer therapy that may be applied to promote cancer susceptibility to TNF-α.

Fig. 3. SH3RF2 regulates PAK4 protein stability. (A and B) Top: immune detection of S3R2 and PAK4 protein in (A) SH3RF2-overexpressing PLC/PRF/5 and SK-Hep1 cells, as well as in (B) SH3RF2 knockdown HeLa cells; bottom: the S3R2 and PAK4 mRNA levels were determined via RT–PCR. S3R2 represents SH3RF2. β-Actin was used as a loading control. (C) The endogenous PAK4 levels were determined in the control and stable SH3RF2 knockdown Huh-7 cells treated with MG132 (10 µM) for 6h. GAPDH was used as a loading control. Quantification of PAK4 protein level analysis was done using NIH Image J software. Error bars, mean ± SD of three independent experiments. (D–F) Ubiquitinated PAK4 protein was detected using anti-HA Ab following immunoprecipitation with anti-Flag M2 affinity gel using the lysates derived from (D) HEK293T cells transfected with PAK4-Flag and Ub-HA constructs and grown with or without MG132 (10 µM) for 6h; (E) HEK293T cells (60 mm dishes) cotransfected with 1 µg of pcDNA/PAK4-Flag, pcDNA/Ub-HA and increasing amounts (1, 2 and 4 µg) of pcDNA/SH3RF2 or empty vector (total amount of transfected vectors = 5 µg) and (F) HEK293T cells cotransfected with pcDNA/PAK4-Flag and pcDNA/Ub-HA vectors together with pcDNA/SH3RF2 or pcDNA/SH3RF2ΔRING vector and treated with MG132 (10 µM) for 6h. GAPDH was used as a loading control.
**Supplementary material**

Supplementary Figures 1–6 and Table 1 can be found at http://carcin.oxfordjournals.org/

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SH3RF2 regulates PAK4 protein stability

Conflict of Interest Statement: None declared.

References


Fig. 5. RNAi-mediated knockdown of SH3RF2 expression results in antitumor effects in vitro and in vivo. (A and B) shControl (shCon.) and stable SH3RF2 knockdown (shSH3RF#2, shSH3RF#3) HeLa cells were used for (A) colony forming assay and (B) in vitro migration assay using a Transwell membrane. S3R2 represents SH3RF2. Error bars, mean ± SD of three independent experiments. *P < 0.05 compared with the control vector. (C) Balb/c nude mice were xenografted with shControl (shCon.) or stable SH3RF2 knockdown (shSH3RF#5) Huh-7 cells. Photographs of tumor tissues extracted on day 20 after injection are shown. (D) Tumor size was determined every other day and the growth patterns are shown graphically.

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