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Tiam1 MUTATIONS IN HUMAN RENAL-CELL CARCINOMAS

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Tiam1 activates the Rho-like GTPase Rac1, and studies indicate that Tiam1–Rac1 signaling affects invasion in different ways depending on the cell type studied. However, no investigations on Tiam1 in human tumors have been reported. Here, we show that for 4 of 5 human renal-cell carcinoma (RCC) cell lines the expression levels of Tiam1 tended to be inversely correlated with *in vitro* invasiveness, whereas no obvious correlation could be found between the expression levels of Rac1 and invasion. Subsequent mutation analysis of these cell lines revealed no mutations in Rac1 but up to 5 different point mutations in the *Tiam1* gene. Of these, 1 mutation (A441G) was located in the NH_2-terminal pleckstrin homology domain, which is essential for membrane localization and functional activity of Tiam1. By analysis of an additional 30 primary human RCCs, mutation A441G was found in 4 of 35 tumors and tumor cell lines (11.5%) but not in the respective normal kidney tissues. By enzymatic digestion, mutation A441G proved to be heterozygous, suggesting a dominant active function. This was supported by showing that stable over-expression of mutated A441G-Tiam1 induced transformation of NIH3T3 cells, as determined in a colony formation assay, whereas empty vector and wild-type Tiam1 failed to do so. In conclusion, a distinct *Tiam1* mutation (A441G) was identified in several human RCCs. This mutation induced transformation of NIH3T3 cells and, hence, might play a major role in the progression of human RCCs. Further analyses on *Tiam1* mutations in human tumors might give new clues to their role in tumor progression.
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Tiam1 has been identified as an invasion- and metastasis-inducing gene in a murine T-lymphoma cell line, and it activates the Rho-like GTPase Rac1 (Habets *et al.,* 1994). Constitutively active Rac1 induces lamellipodia formation and membrane ruffling in fibroblasts and stimulates activation of c-Jun NH_2-terminal kinase (JNK), p38 kinase, NFκB, serum response factor and cyclin D1 promoter, apparently through different independent signaling pathways (Michiels *et al.,* 1995; Westwick *et al.,* 1997; Kheradmand *et al.,* 1998). Over-expression of Tiam1 cDNA in NIH3T3 cells resulted in both membrane ruffling and stimulation of JNK activity through activation of Rac1 (Michiels *et al.,* 1995, 1997). Similar to C-1199-Tiam1, which is an active NH_2-terminally truncated Tiam1 mutant comprising the COOH-terminal 1,199 amino acids (aa), transfection of constitutively active V12Rac1 cDNA induced an oncogenic phenotype in NIH3T3 fibroblasts and *in vitro* invasiveness of murine T-lymphoma cells (van Leeuwen *et al.,* 1995). In contrast, in Ras-transformed Madin-Darby canine kidney (MDCK) cells, Tiam1–Rac1 signaling restored E-cadherin–mediated cell–cell adhesion, resulting in phenotypic reversion and inhibition of invasion in this cell type (Hordijk *et al.,* 1997). This suggests that Rac1 regulates E-cadherin–mediated cell–cell adhesion, which is an important determinant for the invasive capacity of epithelial tumor cells. Invasion and metastasis of carcinoma cells is often associated with reduced E-cadherin–mediated cell–cell adhesion, and reduced expression of, or mutations in, E-cadherin, β-catenin and α-catenin have been identified in human tumors and tumor cell lines (Birchmeier *et al.,* 1995).

The Tiam1 protein comprises 1,591 aa and harbors a number of motifs, the functions of which, however, are only partly known (Habets *et al.,* 1994; Michiels *et al.,* 1997). At the NH_2 terminus of Tiam1, a myristoylation site and 2 PEST domains are present. The latter are thought to be predictors of protein instability but may also be involved in the regulation of protein–protein interactions (Chu *et al.,* 1996). Furthermore, Tiam1 contains a PSD-95-Dlg-ZO-1 (PDZ) domain, also known as Discs-large homologous region. This motif is found in many cell junction–associated proteins implicated in ion-channel and receptor clustering and in coupling proteins to the membrane cytoskeleton (Marfatia *et al.,* 1996). Tiam1 also harbors a Dbl homology (DH) domain and 2 pleckstrin homology (PH) domains, the second of which flanks the DH domain at its COOH terminus. PH domains are protein motifs of about 100 aa which are considered to play an important role in tethering cytosolic proteins to the plasma membrane by protein–protein and/or protein–lipid interactions (Shaw *et al.,* 1996). Point mutations in the PH domain of Bruton′s tyrosine kinase were found to be causative for X-linked agammaglobulinemia (Rawlings *et al.,* 1993; Vihinen *et al.,* 1995). DH domains, in combination with a COOH-terminally flanking PH domain, are characteristic for proteins acting as guanine nucleotide exchange factors (GEFs) for small GTPases of the Rho subfamily (Collard, 1996).

Despite the established role of Tiam1–Rac1 signaling in invasion of murine T-lymphoma cells and Ras-transformed MDCK cells, no investigations on Tiam1 in human tumors have been reported. In the present study, we have analyzed the expression of Tiam1 and Rac1 in 5 different human renal-cell carcinoma (RCC) cell lines in relation to their invasive capacity *in vitro.* Furthermore, we have investigated these cell lines and (as far as available) the respective primary tumors from which they were derived, as well as 30 additional primary human RCCs, for mutations in the *Tiam1* gene. We identified the same *Tiam1* mutation (A441G) in 4 of 35 RCCs investigated and provide evidence that this mutation might play a role in tumor progression.

MATERIAL AND METHODS

Cell lines, cell culture and primary tumors

Five different human RCC cell lines of the clear-cell type (clearCa-5, clearCa-19, clearCa-27, clearCa-28, clearCa-32) were established as previously described (Gerharz *et al.,* 1994; Engers *et al.,* 2000). All RCC cell lines as well as NIH3T3 cells were maintained in DMEM (GIBCO BRL, Karlsruhe, Germany) supplemented with 10% FCS and antibiotics. Cell lines were incubated at 37°C in an atmosphere of 5% CO_2. Primary tumor material, from which RCC cell lines were derived, and corresponding normal kidney tissue of the same patients were available only for clearCa-5 and clearCa-32.

For further analysis of *Tiam1* mutations, paraffin-embedded material of an additional 30 different human clear-cell RCCs with histologically confirmed diagnosis were used.

Matrigel invasion assay

The Matrigel invasion assay imitates active transmigration of tumor cells across a reconstituted basement membrane and was performed as described by Engers *et al.* (1999). Costar (Cambridge, MA) transwells (pore size 8 μm) were coated with Matri-

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gel (Becton Dickinson, Heidelberg, Germany), dried at 37°C in an atmosphere of 5% CO_2 and reconstituted with serum-free medium. The homogeneity of the coating was checked by protein stain. A single-cell suspension of 50,000 tumor cells suspended in serum-free medium was inoculated into the upper chamber, after having added the same medium to the lower chamber. The transmigratory potency of the tumor cells was determined by counting all tumor cells in 5 randomly selected counting areas at the lower surface of the filter after an incubation period of 24 hr. Therefore, cells on the upper surface were wiped away and filters were fixed in methanol and stained with hematoxylin and eosin. Each assay was performed in triplicate and repeated twice.

Immunoprecipitation/Western blotting

Expression of Tiam1 in RCC cell lines was determined by immunoprecipitation: 1×10^7 tumor cells of each cell line were lysed in a buffer containing 150 mM NaCl, 50 mM Tris, 5 mM EDTA, 1% NP40, 20 μM aprotinin, 20 μM leupeptin and 200 μM phenylmethylsulfonyl fluoride. After centrifugation of the lysate, Tiam1 was immunoprecipitated from the detergent-soluble fraction using a polyclonal antibody (Santa Cruz, Heidelberg, Germany) and immunocomplexes were captured by means of protein-A Sepharose beads (Pharmacia, Freiburg, Germany). Beads were washed 3 times in lysis buffer and proteins subsequently released by boiling in SDS sample buffer. Samples were analyzed by SDS-PAGE, and the level of Tiam1 expression was determined by Western blotting using a Tiam1-specific polyclonal antibody (Habets *et al.,* 1994), kindly provided by Dr. J.G. Collard (Amsterdam, the Netherlands), and the enhanced chemiluminescence (ECL) detection system (Amersham, Braunschweig, Germany). Expression of Tiam1 in stably transfected NIH3T3 cells (see below) was analyzed by Western blotting only.

Rac1 expression was monitored by Western blotting using a monoclonal antibody (MAb) kindly provided by Dr. J. Hartwig (Boston, MA). Equal loading of the gels was confirmed by both re-incubation of the same filter with a MAb for α-tubulin (Sigma, Deisenhofen, Germany) and Coomassie blue staining of control gels.

DNA and RNA extraction, RT-PCR and sequence analysis

RNA extraction and RT-PCR. At the time the experiments were performed, no information about the genomic organization of Tiam1 was available. Therefore, initial investigations for Tiam1 and Rac1 mutations were performed with cDNAs. Poly $(A)^+$ RNA was extracted from cell lines and tissues using the Dynabeads mRNA Direct Kit (Dynal, Hamburg, Germany), according to the manufacturer's instructions, and reverse-transcribed using oligo(dT) primers and Moloney murine leukemia virus reverse transcriptase (GIBCO BRL). Water instead of RNA was used as a negative control. PCR was carried out using 50 pmol of each primer, 1 unit Taq DNA polymerase (Hybaid, Heidelberg, Germany) and the Trio-Block Thermal Cycler (Biometra, Göttingen, Germany). The oligonucleotide primers allowing both amplification and sequencing of the entire open reading frames of Tiam1 and Rac1 were obtained from Pharmacia Biotech (Uppsala, Sweden) and Roth (Karlsruhe, Germany). A positive control reaction with actin primers, which amplify a fragment of the human β-actin gene, was tested in a separate PCR. A negative control with water instead of DNA was used for each reaction.

DNA sequencing. PCR products were separated on a 1.5% agarose-TBE gel and eluted by means of the Qiaquick Gel Extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Sequence analyses for both strands were performed using the Taq Dye Deoxynucleotide Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Sequence reactions were run on a DNA sequencer (Applied Biosystems). The sequence was aligned by means of the PC/GENE software package (IntelliGenetics, Mountain View, CA). To exclude PCR artifacts in case of observed mutations, reverse transcriptions, PCRs and subsequent sequence analyses were repeated at least twice.

Extraction of genomic DNA. To investigate the nature of the observed A441G mutation, genomic DNA was isolated from the respective cell lines, primary tumors and corresponding normal kidney tissues using the QIAamp Tissue Kit (Qiagen) according to the manufacturer's instructions. By sequence analysis, human *Tiam1* was shown to harbor an intron between nucleotide (nt) 1884 and nt 1885 (according to the cDNA sequence in the NCBI sequence data library, accession number U16296), which is next to the observed mutation. Using a Tiam1-specific exon–intron primer combination (upstream primer 5′-CTC GTC AGG GGG TGT ACG AG-3′, downstream primer 5′-ACC GGT GCA TTT GGC ACA TAG CCG-3′), spanning codon 441, resulted in a PCR product of 318 bp. The identity of this PCR product as Tiam1 was confirmed by sequence analysis. Since mutation A441G abrogated an EaeI restriction site, the nature of this mutation was investigated by subjecting the respective PCR products to EaeI (Boehringer Mannheim, Mannheim, Germany) restriction digest.

Transfection experiments and colony formation assay

To investigate the functional role of the observed A441G mutation, NIH3T3 cells were stably transfected by retroviral transduction as described (Hordijk *et al.,* 1997) with either empty vector (pLZRS), NH_2-terminally truncated active C-1199-Tiam1 (comprising the COOH-terminal 1,199 aa), full-length Tiam1 (FL-Tiam1) or FL-Tiam1 harboring mutation A441G (FL-A441G-Tiam1). The A441G mutation was introduced by 3-step, PCR-based *in vitro* mutagenesis and verified by sequence analysis. Oligonucleotides for *in vitro* mutagenesis were purchased from Isogen Bioscience (Maarssen, the Netherlands). The mutagenic primer oligonucleotide was 5′-GTT CTT GAC TCC CAG CG-3′. Pools of stably transfected cells were maintained in DMEM supplemented with 10% FCS and antibiotics. G418 was used as a selection marker for the presence of pLZRS in a concentration of 800 μg/ml. To analyze the focus-forming ability of different Tiam1 constructs, transfected cells were grown to confluence and transformed foci were scored 10 days later.

RESULTS

Expression of Tiam1 and Rac1

Expression of Tiam1 and Rac1 was analyzed on both the mRNA (data not shown) and protein levels (Fig. 1*a*). Tiam1 was expressed by 4 of 5 RCC cell lines. The highest level of Tiam1 protein was found in clearCa-19 and clearCa-27, while lower amounts were detected in clearCa-5 and clearCa-32. No expression of Tiam1 was found in clearCa-28, which was confirmed by RT-PCR (data not shown). No major truncations in the Tiam1 protein were detected in these cell lines.

In contrast to Tiam1, Rac1 was detected in all 5 cell lines tested and most strongly expressed by clearCa-5 and clearCa-19, whereas expression levels were lower in clearCa-27, clearCa-32 and clearCa-28. The observed differences in Rac1 expression were not caused by unequal loading of the gel, as confirmed by both re-incubation of the same filter with an antibody against α-tubulin and Coomassie blue staining of control gels.

In vitro *invasiveness*

In the Matrigel invasion assay, marked differences in the invasive behavior of the cell lines were found (Fig. 1*b*). While clearCa-28 and clearCa-5 were strongly invasive, clearCa-19 exhibited an intermediate and clearCa-27 and clearCa-32 a low invasive potential.

When compared to expression levels of Tiam1 and Rac1, the 2 most invasive cell lines either failed to express Tiam1 (clearCa-28) or exhibited very low expression levels of Tiam1 (clearCa-5). Conversely, expression levels of Tiam1 in clearCa-19 and clearCa-27, which are of intermediate or low invasive potential, respectively, were markedly higher. These results are in line with the known anti-invasive effect of Tiam1 in Ras-transformed MDCK cells (Hordijk *et al.,* 1997) and suggest that down-regulation of Tiam1 might contrib-

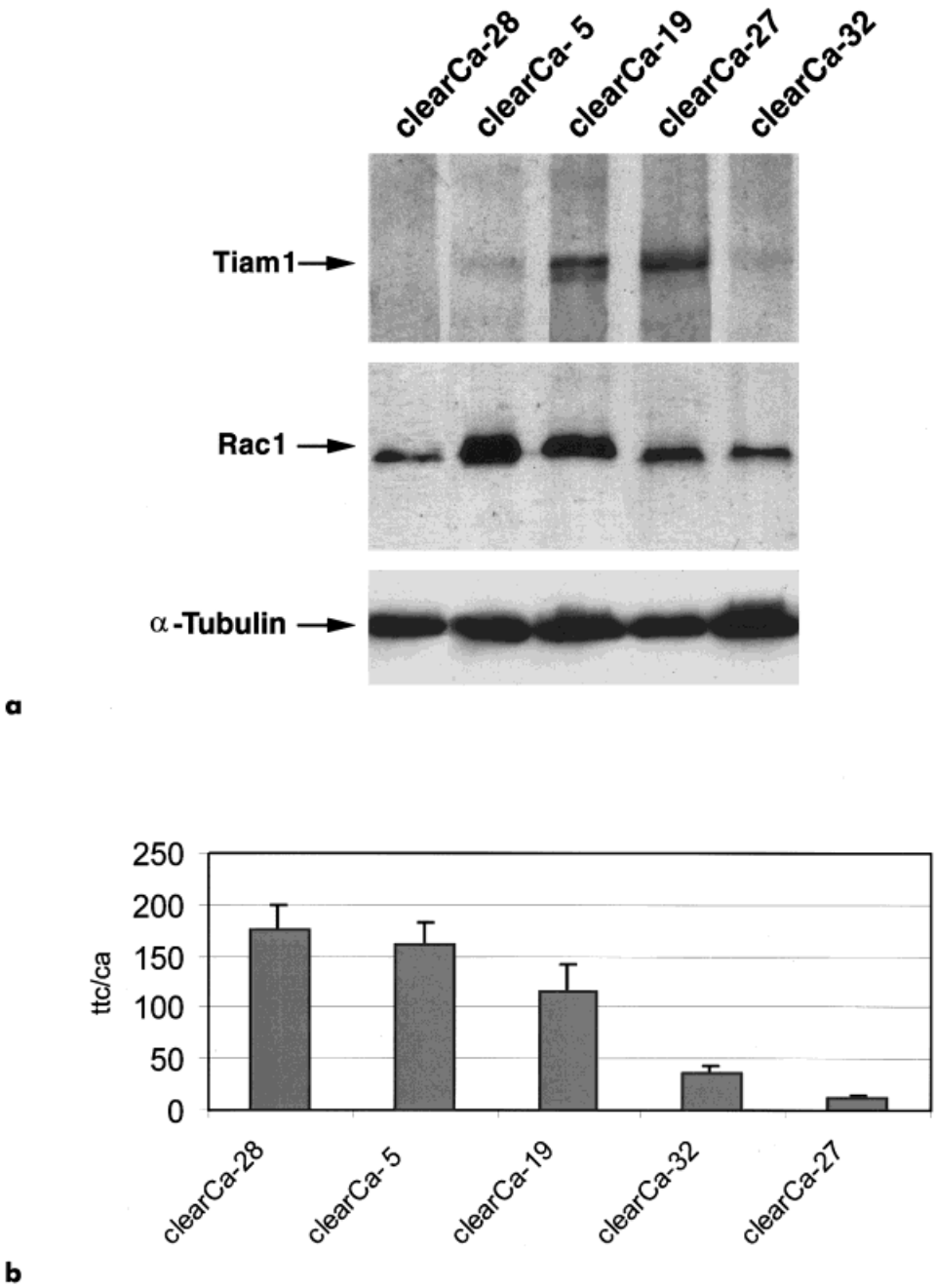


FIGURE 1 – *(a)* Protein expression of Tiam1 and Rac1 in 5 human RCC cell lines as determined by combined immunoprecipitation and immunoblotting (Tiam1) or immunoblotting only (Rac1). The lack of Tiam1 expression in clearCa-28 has been confirmed by RT-PCR (data not shown). Differences in Rac1 expression were not due to unequal protein loading, as determined by re-incubation of the same filter with an antibody against α-tubulin and Coomassie blue staining of control gels. Results are representative for 3 independent experiments. *(b) In vitro* invasiveness of 5 human RCC cell lines of the clear-cell type, as determined by Matrigel invasion assay after 24 hr. Major differences in invasion were detected among the different cell lines (ttc/ca, number of transmigrated tumor cells/counting area). Experiments were performed in triplicate and repeated twice.

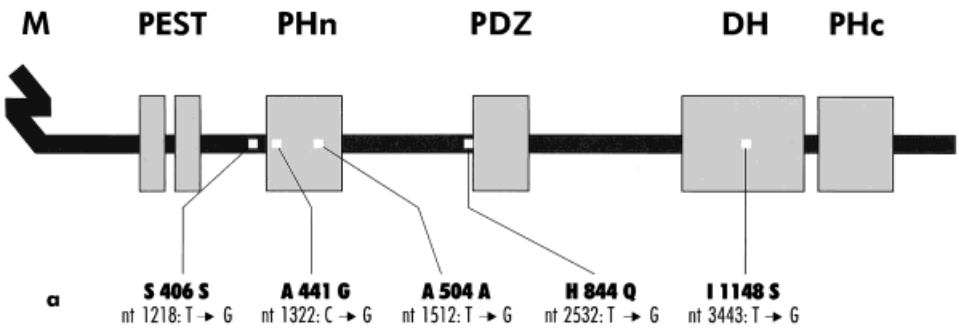
ute to the invasive phenotype of human RCCs. However, the least invasive cell line, clearCa-32, expressed only very low amounts of Tiam1, suggesting that down-regulation of Tiam1 is not sufficient to induce a strong invasive phenotype in human RCC. Moreover, no obvious correlation was seen between *in vitro* invasiveness of all 5 cell lines and expression levels of Rac1.

Analysis of Tiam1 and Rac1 genes for mutations

Since not only expression levels but also functional activities determine the biological role of proteins, the *Tiam1* and *Rac1* genes were analyzed for the presence of mutations. As intron sequences of both genes were initially unknown, these investigations were started on the cDNA level. By sequence analysis of the entire open reading frame of *Rac1*, no mutations in this gene could be detected in any of the 5 RCC cell lines investigated (Fig. 2).

In contrast, the 4 Tiam1-expressing RCC cell lines were found to harbor between 2 and 5 different point mutations in the *Tiam1* gene (Fig. 2). Each mutation was detected in at least 2 different cell lines, and the entire open reading frame of Tiam1, consisting

a



b

	Staging and grading of original tumors	Tiam1 mutations					Rac1 mutations
		S 406 S	A 441 G	A 504 A	H 844 Q	I 1148 S	
clearCa-5	pT3b, G3	+	-	+	+[1]	-	-
clearCa-19	pT2, G2	+	+	+	+	-	-
clearCa-27	pT3a, G3	-	-	-	+	+	-
clearCa-28	pT2, G3	n.a.	n.a.	n.a.	n.a.	n.a.	-
clearCa-32	pT3a, G2	+	+[2]	+	+[1]	+[2]	-

c

number of additional primary RCCs investigated	number of Tiam1-expressing RCCs	number of A441G mutations
30	30	2

d

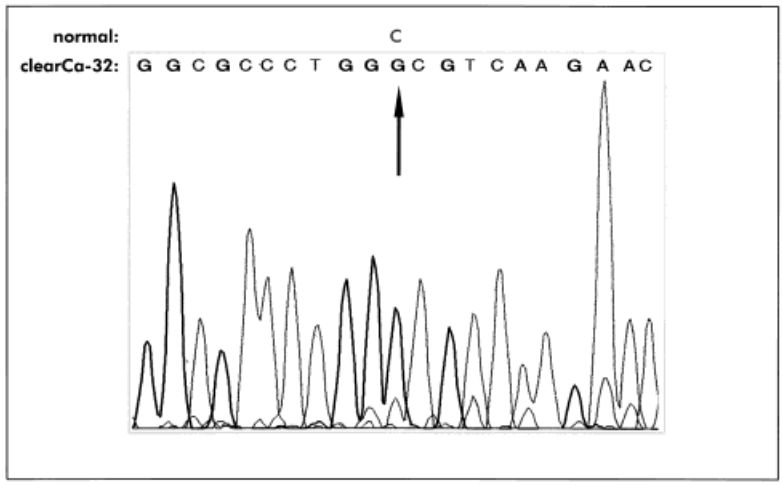


FIGURE 2 – Analysis of human RCCs for Tiam1 and Rac1 mutations. *(a)* Domain structure of the Tiam1 protein and localization of observed mutations. *(b)* List of mutations found in the human RCC cell lines. "Staging and grading" refers to the extent and degree of differentiation, respectively, of primary tumors. Cell line clearCa-28 did not express Tiam1 and therefore was not analyzed (n.a.) for Tiam1 mutations. Primary tumors and corresponding normal kidney tissues were available only for clearCa-5 and clearCa-32. [1)]Mutation also present in both the primary tumor, from which the cell line was derived, and the corresponding normal kidney tissue. [2)]Mutation also present in the primary tumor, from which the cell line was derived, but not in the corresponding normal kidney tissue. *(c)* Analysis of an additional 30 human RCCs for the presence of mutation A441G in the *Tiam1* gene. *(d)* Representative genomic sequence of Tiam1 in clearCa-32, presented in the 5′ to 3′ direction, with a point mutation (C→G) in codon 441, resulting in a substitution of alanine (GCC) by glycine (GGC).

of 1,591 aa, was sequenced. These mutations were found by sequence analysis of at least 3 independently generated PCR products, excluding PCR-based artifacts. Only 3 of these mutations (A441G, H844Q and I1148S) resulted in a change of the aa code and, therefore, were considered to be of possible functional importance. These mutations were located either in the PHn domain (A441G), next to the PDZ domain (H844Q) or in the catalytic DH domain (I1148S) (Fig. 2). In particular, mutations in the PHn domain and the DH domain could be of interest because small deletions in these domains were shown to inhibit the functional

activity of Tiam1 (Michiels *et al.,* 1997). To identify cell culture artifacts and polymorphisms, both primary tumors and the respective normal kidney tissues, which were available for clearCa-5 and clearCa-32, were analyzed for the presence of these mutations. Mutation H844Q was also detected in both primary tumors and corresponding normal kidney tissues of clearCa-5 and clearCa-32, indicating that this mutation represents a polymorphism rather than a functionally important mutation. In contrast, mutations I1148S and A441G were observed in the primary tumor of clearCa-32 but not in the respective normal kidney tissue, excluding a cell culture artifact or a polymorphism. Sequence comparison between human and murine Tiam1 (Habets *et al.,* 1995), however, revealed that serine-1148 is the normal aa of murine Tiam1, suggesting that this mutation is not likely to be of functional importance.

In contrast, mutation A441G was located in the PHn domain of Tiam1, which is essential for functional activity of the protein (Michiels *et al.,* 1997), and affected an aa that was evolutionarily conserved between mouse and human Tiam1. For more information about the frequency of A441G mutations in human RCCs, an additional 30 primary tumors were analyzed for the presence of this mutation. Interestingly, 2 of these 30 tumors also proved to harbor mutation A441G, whereas this mutation was absent in the corresponding normal kidney tissues (Fig. 2). Thus, mutation A441G was restricted to tumor tissues and found in 4 of 35 RCCs (11.5%).

Since mutation A441G affected an EaeI restriction site in the *Tiam1* gene, the nature of this mutation was investigated by enzymatic digest with EaeI (Fig. 3). After having identified and sequenced an intron next to the observed Tiam1 mutation (data not shown), PCR products of 318 bp, spanning codon 441, were amplified from genomic DNA. EaeI digest of PCR products derived from normal kidney tissues were completely digested into 2 fragments, 187 and 131 bp, by EaeI. These results confirm the presence of an EaeI restriction site in both alleles of normal human *Tiam1* and, thus, the absence of mutation A441G. In contrast, the respective tumor-derived PCR products harboring mutation A441G were only partly digested by EaeI into the same fragments, indicating the heterozygous nature of this mutation.

Functional role of Tiam1 *mutation A441G*

Mutation A441G was located in the PHn domain of Tiam1, which is essential for membrane localization and functional activity of the Tiam1 protein as shown in NIH3T3 cells (Michiels *et al.,* 1997). To explore the role of this mutation, 3 different Tiam1 cDNA constructs (FL-Tiam1, FL-A441G-Tiam1 and C-1199-Tiam1) were stably over-expressed in NIH3T3 cells and the effects on cell morphology and ability to form colonies analyzed. Transfection of either of the different Tiam1 cDNAs induced membrane ruffling and an epithelial phenotype, suggesting that these biological properties are not affected by mutation A441G (Fig. 4). The effects of FL-Tiam1 and FL-A441G-Tiam1 on cell morphology were somewhat less pronounced than those of C-1199-Tiam1, which is in accordance with earlier reports (Michiels *et al.,* 1995, 1997; Hordijk *et al.,* 1997; Sander *et al.,* 1998). However, marked differences were observed between the different Tiam1 cDNAs in their ability to induce colony formation in NIH3T3 cells. In line with previous observations (van Leeuwen *et al.,* 1995), neither mock- nor FL-Tiam1-transfected cells were capable of forming colonies 10 days after confluence had been reached, whereas numerous colonies were seen in C-1199-Tiam1–expressing cells (Fig. 4). In contrast to FL-Tiam1, ectopic expression of FL-A441G-Tiam1 induced colony formation to a similar extent as C-1199-Tiam1 (Fig. 4). Since colony formation in NIH3T3 cells reflects transformation, our results suggest that *Tiam1* mutation A441G is sufficient to transform NIH3T3 cells and consequently might play an important role in the progression of human RCCs.

DISCUSSION

The GEF protein Tiam1 was initially identified as an invasion- and metastasis-inducing gene in a murine T-lymphoma cell line that activates the Rho-like GTPase Rac1 (Habets *et al.,* 1994; Michiels *et al.,* 1995). In contrast, in Ras-transformed MDCK cells, Tiam1 and Rac1 inhibit hepatocyte growth factor–induced scattering and invasion into collagen by promoting E-cadherin–mediated cell–cell adhesion (Hordijk *et al.,* 1997), suggesting a cell type-specific role of Tiam1–Rac1 signaling in invasion.

No investigations on Tiam1 in human tumors have been reported, which might be due to the fact that Tiam1-specific antibodies that would allow immunohistochemical studies on paraffin-embedded material, are not available. In the present study, 5 human RCC cell lines were characterized for expression of Tiam1 and Rac1 in relation to their invasive potential *in vitro.* In the Matrigel invasion assay, imitating active transmigration of tumor cells across a reconstituted basement membrane, major differences in *in vitro* invasiveness were found among the different cell lines.

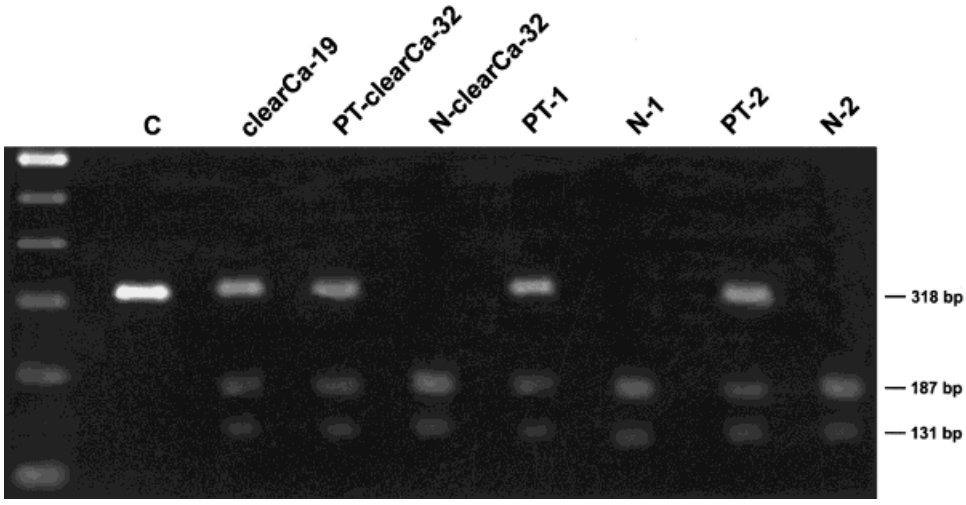


FIGURE 3 – Analysis of the nature of mutation A441G by EaeI digestion. PCR products of 318 bp spanning codon 441 were amplified from genomic DNA of cell line clearCa-19, primary tumors (PT-clearCa-32, PT-1, PT-2) and the respective normal kidney tissues (N-clearCa-32, N-1, N-2) and subjected to EaeI digestion. A non-digested PCR product was used as control (C). Whereas PCR products derived from normal tissues were entirely digested into 2 fragments, 187 and 131 bp, tumor-derived PCR products harboring mutation A441G were only partly digested into the same products, indicating the heterozygous nature of this mutation.

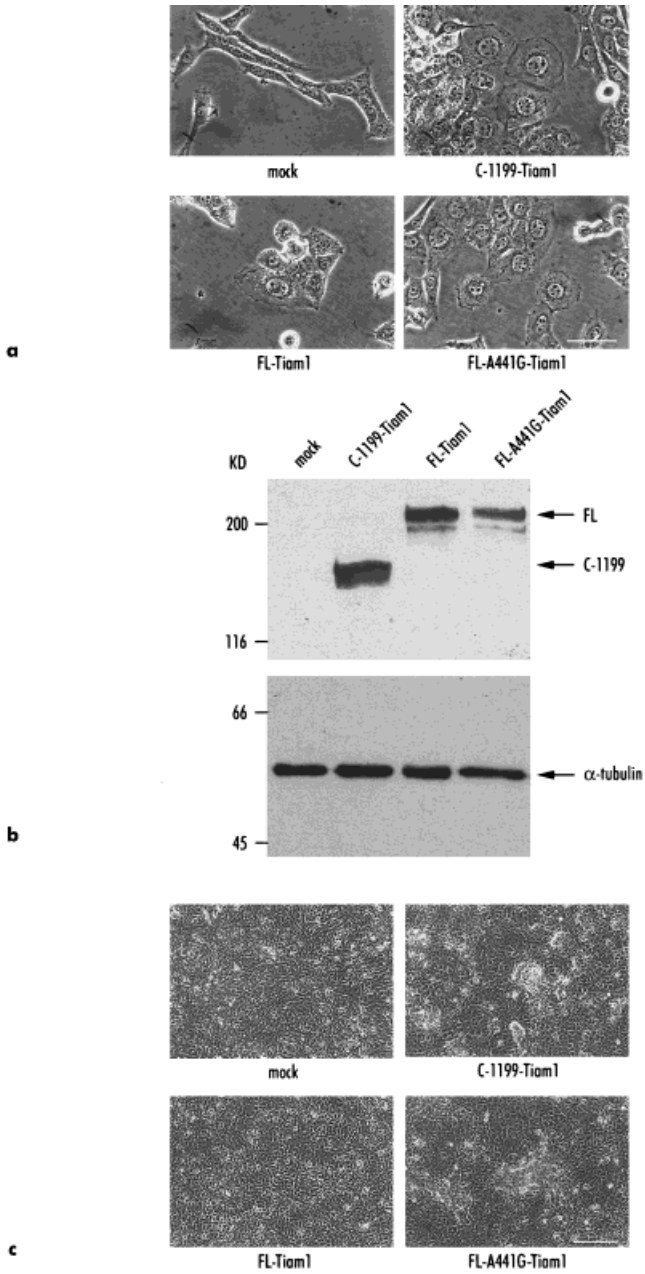


FIGURE 4 – Functional analysis of Tiam1 mutation A441G in NIH3T3 cells. *(a)* Morphology of NIH3T3 cells stably transfected with different Tiam1 constructs and analyzed by phase-contrast microscopy. Mock-transfected cells are spindle-shaped, exhibit a mesenchymal phenotype and do not show membrane ruffling. Cells transfected with either C-1199-Tiam1, FL-Tiam1 or FL-A441G-Tiam1 are large and flat, show pronounced membrane ruffling and exhibit an epithelial phenotype. No marked morphological differences are observed between cells expressing FL-Tiam1 or FL-A441G-Tiam1. All pictures were taken at the same magnification. Scale bar = 20 μm. *(b)* Immunodetection of Tiam1 proteins in NIH3T3 cells upon stable transfection with different Tiam1 constructs. Results are representative of 3 independent experiments. *(c)* Colony formation in stably transfected NIH3T3 cells. Cells were grown to confluence and colonies scored 10 days later. Whereas no colonies were detectable in mock- and FL-Tiam1–transfected cells, numerous colonies were observed in cells expressing C-1199-Tiam1 or FL-A441G-Tiam1. All pictures were taken at the same magnification. Scale bar = 70 μm.

For 4 of 5 cell lines, the invasive potential tended to be inversely correlated to the expression levels of Tiam1. These results are in line with the known anti-invasive function of Tiam1 in Ras-transformed MDCK cells (Hordijk *et al.,* 1997) and suggest that RCC invasiveness might partly result from down-regulation of Tiam1. The fact, however, that the least invasive cell line, clearCa-32, expressed only very low amounts of Tiam1 suggests that down-regulation of Tiam1 is not sufficient to induce a strong invasive phenotype in human RCC. In contrast to Tiam1, no correlation between the expression levels of Rac1 and invasiveness was observed.

Based on the established role of Tiam1–Rac1 signaling in tumor invasion, we then investigated whether mutations of both genes occurred in human RCC. Similar to Ras, distinct point mutations of Rac1 result in constitutive activation of the protein (Ridley *et al.,* 1992; Westwick *et al.,* 1997). Therefore, all 5 RCC cell lines were analyzed for the presence of Rac1 mutations. However, analyzing the entire open reading frame of Rac1, no mutations were detected in any of the cell lines tested.

For GEF proteins like Dbl, Ost, Vav, Ect2, Tim and Lbc, NH_2-terminal truncations have been found to activate the respective protein (Miki *et al.,* 1993; Chan *et al.,* 1994; Horii *et al.,* 1994; Khosravi *et al.,* 1994; Toksoz and Williams *et al.,* 1994). Although NH_2-terminal truncation resulting from retroviral integration is known to activate Tiam1 (Habets *et al.,* 1994), no naturally occurring Tiam1 truncations were detected in the RCC cell lines. However, by sequence analysis of the entire coding sequence of Tiam1, up to 5 different point mutations were found in all 4 Tiam1-expressing RCC cell lines. Only 3 of these mutations, located within the PHn (A441G), DH (I1148S) or next to the PDZ (H844Q) domain, respectively, affected the aa code and, therefore, were considered to be of possible functional importance. Sequence analysis of primary tumors from which the cell lines were derived and the respective normal kidney tissues, however, revealed that mutation H844Q represents a polymorphism, whereas mutations I1148S and A441G were exclusively found in tumor tissues. Despite its tumor-restricted detection, mutation I1148S appears unlikely to be of functional importance since sequence comparison between human and murine Tiam1 (Habets *et al.,* 1995) revealed that serine-1148 is the normal aa of murine Tiam1 and over-expression of murine Tiam1 cDNAs in human cells induces phenotypes similar to those reported for non-human cells (data not shown). In contrast, mutation A441G, located in the PHn domain and present in 2 of the RCC cell lines, might be functionally relevant. This mutation was also detected in the primary tumor of clearCa-32 (primary tumor material of the second cell line was not available) but not in the corresponding normal kidney tissue of the same patient. Moreover, mutation A441G was also found in 2 of 30 additionally investigated primary RCCs and proved to be absent in the corresponding normal kidney tissues. These results excluded both a polymorphism and a cell culture artifact and suggested a possible role of the A441G mutation. By enzymatic digestion, mutation A441G was found to be heterozygous, suggesting a dominant active function. A functional role of this mutation in the regulation of invasion of human RCCs, however, appears unlikely since no correlation with the invasive phenotype of our cell lines was observed.

In NIH3T3 cells, over-expression of Tiam1 induces morphological alterations and NH_2-terminal truncations of Tiam1 result in transformation and oncogenic activation (van Leeuwen *et al.,* 1995). We therefore investigated whether some of these biological properties were affected by mutation A441G. In contrast to active C-1199-Tiam1, stable transfection of FL-Tiam1 was not sufficient to induce colony formation in NIH3T3 cells, which is in line with earlier observations (van Leeuwen *et al.,* 1995). However, FL-A441G-Tiam1–transfected cells gave rise to numerous colonies, comparable to cells transfected with C-1199-Tiam1. Since colony formation in NIH3T3 cells reflects transformation, we conclude that mutation A441G confers transforming activity to the *Tiam1*

gene and, hence, might play a major role in the progression of human RCCs. In contrast to colony formation, mutation A441G did not affect the ability of Tiam1 to induce membrane ruffling and an epithelial phenotype in NIH3T3 cells. This suggests that these different biological properties of Tiam1 are regulated by different signaling pathways. So far, Tiam1 has been reported to activate only Rac1, and Rac1 in turn is known to activate at least 5 different signaling pathways (Westwick *et al.,* 1997; Kheradmand *et al.,* 1998). It is conceivable that Tiam1, as a Rac1 exchanger, determines the binding of specific Rac1 effectors, as found for the exchanger PIX (Manser *et al.,* 1998); hence, mutation A441G might affect 1 or several Rac1-dependent signaling pathways.

Moreover, mutation A441G might affect the interaction of the PHn domain of Tiam1 with distinct upstream regulating proteins. When compared to other PH domains, this mutation affects an aa that is conserved between Tiam1 and Dbl and only 1 aa downstream of a lysine, which is conserved in at least 12 different PH domains (Habets *et al.,* 1994). When compared to the PH domains of pleckstrin, spectrin or phospholipase C-δ1, mutation A441G in Tiam1 is located immediately upstream of the binding sites for inositol phosphates and next to the protein kinase C–binding sites of the PH domains of Bruton′s tyrosine kinase and rac protein kinases (Yao *et al.,* 1994; Konishi *et al.,* 1994; Ferguson *et al.,* 1995; Salim *et al.,* 1996). Binding of the PHn domain of Tiam1 to distinct inositol phosphates and phosphorylation of Tiam1 by protein kinase C have also been reported (Fleming *et al.,* 1998; Rameh *et al.,* 1997), but the respective specific binding or phosphorylation sites have not been identified. Thus, it remains to be determined whether these interactions are affected by the observed A441G mutation.

In conclusion, we identified a distinct *Tiam1* mutation (A441G) in several human RCCs. This mutation was sufficient to transform NIH3T3 cells in a colony formation assay and, hence, might play a major role in the progression of human RCCs. Given the present results and the established importance of Tiam1 and Rac1 in invasion of different cell types, a more extensive analysis on mutations in Tiam1 and Rac1 in human tumors might give new clues about their function in tumor progression.

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REFERENCES

Birchmeier, W., Hulsken, J. and Behrens, J., Adherens junction proteins in tumour progression. *Cancer Surv.,* **24,** 129–140 (1995).

Chan, A.M., McGovern, E.S., Catalano, G., Fleming, T.P. and Miki, T., Expression cDNA cloning of a novel oncogene with sequence similarity to regulators of small GTP-binding proteins. *Oncogene,* **9,** 1057–1063 (1994).

Chu, Z.L., Mckinsey, T.A., Liu, L., Qi, X. and Ballard, D.W., Basal phosphorylation of the PEST domain in the I(kappa)B(beta) regulates its functional interaction with the c-rel proto-oncogene product. *Mol. cell. Biol.,* **16,** 5974–5984 (1996).

Collard, J.G., Signaling pathways regulated by Rho-like proteins: a possible role in tumor formation and metastasis. *Int. J. Oncol.,* **8,** 131–138 (1996).

Engers, R., Gerharz, C.D., Donner, A., Mrzyk, S., Krause-Paulus, R., Petek, O. and Gabbert, H.E., *In vitro* invasiveness of human epithelioid-sarcoma cell lines: association with cell motility and inverse correlation with the expression of tissue inhibitor of metalloproteinases. *Int. J. Cancer,* **80,** 406–412 (1999).

Engers, R., Mrzyk, S., Springer, E., Fabbro, D., Weissgerber, G., Gerharz, C.D. and Gabbert, H.E., Protein kinase C in human renal cell carcinomas: role in invasion and differential isoenzyme expression. *Brit. J. Cancer,* **82,** 1063–1069 (2000).

Ferguson, K.M., Lemmon, M.A., Schlessinger, J. and Sigler, P.B., Structure of the high affinity complex of inositol trisphosphate with a phospholipase C pleckstrin homology domain. *Cell,* **83,** 1037–1046 (1995).

Fleming, I.N., Elliott, C.M. and Exton, J.H., Phospholipase C-gamma, protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase II are involved in platelet-derived growth factor–induced phosphorylation of Tiam1. *FEBS Lett.,* **429,** 229–233 (1998).

Gerharz, C.D., Ramp, U., Olert, J., Moll, R., Storkel, S., Marx, N. and Gabbert, H.E., Cytomorphological, cytogenetic, and molecular biological characterization of four new human renal carcinoma cell lines of the clear cell type. *Virchows Arch.,* **424,** 403–409 (1994).

Habets, G.G., Scholtes, E.H., Zuydgeest, D., van der Kammen, R.A., Stam, J.C., Berns, A. and Collard, J.G., Identification of an invasion-inducing gene, *Tiam-1,* that encodes a protein with homology to GDP–GTP exchangers for Rho-like proteins. *Cell,* **77,** 537–549 (1994).

Habets, G.G., van der Kammen, R.A., Stam, J.C., Michiels, F. and Collard, J.G., Sequence of the human invasion-inducing *TIAM1* gene, its conservation in evolution and its expression in tumor cell lines of different tissue origin. *Oncogene,* **10,** 1371–1376 (1995).

Hordijk, P.L., ten Klooster, J.P., van der Kammen, R.A., Michiels, F., Oomen, L.C. and Collard, J.G., Inhibition of invasion of epithelial cells by Tiam1-Rac signaling. *Science,* **278,** 1464–1466 (1997).

Horii, Y., Beeler, J.F., Sakaguchi, K., Tachibana, M. and Miki, T., A novel oncogene, *ost,* encodes a guanine nucleotide exchange factor that potentially links Rho and Rac signaling pathways. *EMBO J.,* **13,** 4776–4786 (1994).

Kheradmand, F., Werner, E., Tremble, P., Symons, M. and Werb, Z., Role of Rac1 and oxygen radicals in collagenase-1 expression induced by cell shape change. *Science,* **280,** 898–902 (1998).

Khosravi, F.R., Chrzanowska, W.M., Solski, P.A., Eva, A., Burridge, K. and Der, C.J., Dbl and Vav mediate transformation *via* mitogen-activated protein kinase pathways that are distinct from those activated by oncogenic Ras. *Mol. cell. Biol.,* **14,** 6848–6857 (1994).

Konishi, H., Shinomura, T., Kuroda, S., Ono, Y. and Kikkawa, U., Molecular cloning of rat RAC protein kinase alpha and beta and their association with protein kinase C zeta. *Biochem. biophys. Res. Comm.,* **205,** 817–825 (1994).

Manser, E., Loo, T.H., Koh, C.G., Zhao, Z.S., Chen, X.Q., Tan, L., Tan, I., Leung, T. and Lim, L., PAK kinases are directly coupled to the PIX family of nucleotide exchange factors. *Mol. Cell,* **1,** 183–192 (1998).

Marfatia, S.M., Cabral, J.H., Lin, L., Hough, C., Bryant, P.J., Stolz, L. and Chishti, A.H., Modular organization of the PDZ domains in the human discs—large protein suggests a mechanism for coupling PDZ domain-binding proteins to ATP and the membrane cytoskeleton. *J. Cell Biol.,* **135,** 753–766 (1996).

Michiels, F., Habets, G.G., Stam, J.C., van der Kammen, R.A. and Collard, J.G., A role for Rac in Tiam1-induced membrane ruffling and invasion. *Nature (Lond.),* **375,** 338–340 (1995).

Michiels, F., Stam, J.C., Hordijk, P.L., van der Kammen, R.A., Ruuls-van Stalle, L., Feltkamp, C.A. and Collard, J.G., Regulated membrane localization of Tiam1, mediated by the NH_2-terminal pleckstrin homology domain, is required for Rac-dependent membrane ruffling and C-Jun NH_2-terminal kinase activation. *J. Cell Biol.,* **137,** 387–398 (1997).

Miki, T., Smith, C.L., Long, J.E., Eva, A. and Fleming, T.P., Oncogene *ect2* is related to regulators of small GTP-binding proteins [published erratum appears in *Nature (Lond.),* **364,** 737 (1993)]. *Nature (Lond.),* **362,** 462–465 (1993).

Rameh, L.E. and 12 others, A comparative analysis of the phosphoinositide binding specificity of pleckstrin homology domains. *J. biol. Chem.,* **272,** 22059–22066 (1997).

Rawlings, D.J., Saffran, D.C., Tsukada, S., Largaespada, D.A., Grimaldi, J.C., Cohen, L., Mohr, R.N., Bazan, J.F., Howard, M., Copeland, N.G. and Et, A., Mutation of unique region of Bruton's tyrosine kinase in immunodeficient XID mice. *Science,* **261,** 358–361 (1993).

Ridley, A.J., Paterson, H.F., Johnston, C.L., Diekmann, D. and Hall, A., The small GTP-binding protein rac regulates growth factor–induced membrane ruffling. *Cell,* **70,** 401–410 (1992).

SALIM, K., BOTTOMLEY, M.J., QUERFURTH, E., ZVELEBIL, M.J., GOUT, I., SCAIFE, R., MARGOLIS, R.L., GIGG, R., SMITH, C.I., DRISCOLL, P.C., WATERFIELD, M.D. and PANAYOTOU, G., Distinct specificity in the recognition of phosphoinositides by the pleckstrin homology domains of dynamin and Bruton's tyrosine kinase. *EMBO J.,* **15,** 6241–6250 (1996).

SANDER, E.E., VAN DELFT, S., TEN KLOOSTER, J.P., REID, T., VAN DER KAMMEN, R.A., MICHIELS, F. and COLLARD, J.G., Matrix-dependent Tiam1/Rac signaling in epithelial cells promotes either cell–cell adhesion or cell migration and is regulated by phosphatidylinositol 3-kinase. *J. Cell Biol.,* **143,** 1385–1398 (1998).

SHAW, G., The pleckstrin homology domain: an intriguing multifunctional protein module. *Bioessays,* **18,** 35–46 (1996).

TOKSOZ, D. and WILLIAMS, D.A., Novel human oncogene *lbc* detected by transfection with distinct homology regions to signal transduction products. *Oncogene,* **9,** 621–628 (1994).

VAN LEEUWEN, F.N., VAN DER KAMMEN, R.A., HABETS, G.G. and COLLARD, J.G., Oncogenic activity of Tiam1 and Rac1 in NIH3T3 cells. *Oncogene,* **11,** 2215–2221 (1995).

VIHINEN, M., ZVELEBIL, M.J., ZHU, Q., BROOIMANS, R.A., OCHS, H.D., ZEGERS, B.J., NILSSON, L., WATERFIELD, M.D. and SMITH, C.I., Structural basis for pleckstrin homology domain mutations in X-linked agammaglobulinemia. *Biochemistry,* **34,** 1475–1481 (1995).

WESTWICK, J.K., LAMBERT, Q.T., CLARK, G.J., SYMONS, M., VAN AELST, L., PESTELL, R.G. and DER, C.J., Rac regulation of transformation, gene expression, and actin organization by multiple, PAK-independent pathways. *Mol. cell. Biol.,* **17,** 1324–1335 (1997).

YAO, L., KAWAKAMI, Y. and KAWAKAMI, T., The pleckstrin homology domain of Bruton tyrosine kinase interacts with protein kinase C. *Proc. Nat. Acad. Sci. (Wash.),* **91,** 9175–9179 (1994).