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Rac1 in human breast cancer: overexpression, mutation analysis, and characterization of a new isoform, Rac1b

A Schnelzer[1], D Prechtel[2], U Knaus[3], K Dehne[1], M Gerhard[4], H Graeff[1], N Harbeck[1], M Schmitt[1] and E Lengyel*,[1]

[1]*Department of Obstetrics and Gynecology, Technische Universität München, Klinikum rechts der Isar, Ismaninger Str. 22, 0-81675 Munich, Germany;* [2]*Department of Pathology, Technische Universität München, Klinikum rechts der Isar, 0-81675 Munich, Germany;* [3]*Department of Immunology, The Scripps Research Institute, La Jolla, California, CA 92037, USA;* [4]*Department of Internal Medicine II, Technische Universität München, Klinikum rechts der Isar, 81675 Munich, Germany*

Rac1 is a member of the Ras superfamily of small guanosine triphosphatases (GTPases) that act as molecular switches to control cytoskeletal rearrangements and cell growth. Analogous to Ras, constitutively activating point mutations of Rac1 cause tumorigenic transformation of cell lines. However, there is no information about whether Rac1 is also mutated *in vivo*. After RT–PCR of Rac1, several clones of seven benign and 10 malignant breast cancer tissues as well as eight breast cancer cell lines were sequenced. Only single-nucleotide polymorphisms of Rac1 could be detected, and none of these corresponded to constitutively activating point mutations that have been used in cell lines for transformation. While sequencing Rac1 in breast tissues, a new Rac1 isoform with an insertion of 19 codons within the reading frame of Rac1 close to switch region II was identified and named Rac1b. The Rac1b protein acts like a fast cycling GTPase in GTP binding and hydrolysis assays. In Northern and Western blot experiments both Rac1 RNA and Rac1 protein had a significantly higher expression in breast cancer tissues compared to normal breast tissue samples. Immunohistochemical staining of Rac1 showed weak Rac1 expression in benign breast disease but high expression level in ductal carcinoma-in-situ, primary breast cancer, and lymph node metastases. In addition, breast tumor cells from patients with recurrent disease had Rac1 expression at the plasma membrane, suggesting activation of Rac1, in patients with aggressive breast cancer. *Oncogene* (2000) **19,** 3013–3020.

Keywords: Rho GTPases; Rac1; isoform; breast cancer; ductal carcinoma-in-situ; metastasis

Introduction

The Rho family of small guanosine triphosphatases (GTPases) forms a subgroup of the Ras superfamily of GTP-binding proteins and consists of 12 mammalian members. Until recently, their main cellular functions seemed to be reorganization of the actin cytoskeleton and cell adhesion in response to stimulation by growth factors. There is now increasing evidence that Rho GTPases also play a role in cell cycle regulation and membrane trafficking (Zohn *et al*., 1998; Hall, 1998). Further, Rac1 and Cdc42 participate in signal transduction pathways that activate the c-Jun N-terminal and the p38 mitogen-activated protein kinase (MAPK) pathway (Coso *et al*., 1995). GTP-binding proteins exist in two conformational states, an inactive GDP-bound form and an active GTP-bound form, and their interconversion occurs through a cycle of guanine exchange and GTP hydrolysis (Zohn *et al*., 1998). Guanine nucleotide exchange factors convert GTP-binding proteins to their active GTP-bound form, while GTPase-activating proteins stimulate their intrinsic GTPase activity, inactivating the Rho GTPase. Activating mutations in Rho GTPases inhibit the intrinsic GTPase rate and block GAP-stimulated GTP hydrolysis (Khosravi-Far *et al*., 1995).

In cultured cells, Rho GTPases play an important part in several aspects of cell proliferation. For instance, a GTPase-deficient constitutively activated mutant of Rac1 (Rac1 V12), analogous to an activating Ras mutation (Bos, 1989), caused DNA synthesis in quiescent Swiss 3T3 fibroblasts. Qiu *et al*. showed that Rat1 fibroblasts expressing activated Rac1 V12 display all hallmarks of malignant transformation (Qiu *et al*., 1995). Rac1 V12 overexpressing cells had a higher growth rate, lost contact inhibition, and induced tumors in nude mice. Activated Rac1 and RhoA further enhanced oncogenic Ras-triggered morphologic transformation, as well as growth in soft agar (Khosravi-Far *et al*., 1995). Stable transfection of a dominant-negative Rac1 (Rac1 N17) inhibited Ras-induced focus formation, indicating that Rac1 is necessary for Ras transformation (Khosravi-Far *et al*., 1995).

All published studies that characterize the transforming potential of Rac1 were performed *in vitro* using constitutively activated forms of Rac1 (Rac1 V12, Rac1 L61, Rac1 I115) and mainly murine fibroblast cell lines (Khosravi-Far *et al*., 1995; Qiu *et al*., 1995; Anand-Apte *et al*., 1997; Keely *et al*., 1997). While tissue culture is an appropriate system to characterize the function and regulation of Rac1 as related to cell lines, there is no evidence in human tumors that Rac1 contributes to malignancy or that activating mutations occur *in vivo*. However, studies on Ras showed that activating Ras mutations characterized in cell culture are present in cancer tissue and are important for malignant transformation (Bos, 1989).

With this in mind, we undertook a study to determine whether there are any mutations present in the Rac1 gene in breast cancer tissue cells and to analyse whether Rac1 expression is altered during malignant progression. Breast cancer is one of the most

*Correspondence: E Lengyel
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common malignancies in the Western world, and has a high mortality rate (Harbeck *et al*., 1999). The etiology of the disease remains unknown, and aside from a small subset of patients (~5%) with hereditary genetic alterations, sporadic breast cancer accounts for the majority of all breast cancers. Several studies (Dickson *et al*., 1992; Sivaraman *et al*., 1997) suggested a prominent role for the MAPK regulatory network in the pathogenesis of breast cancer which cannot be explained by mutations in the Ras gene only, as Ras mutations only occur in 8% of breast cancer patients (Bos, 1989). Therefore, we were interested in investigating whether other GTPases like Rac1 are mutated or overexpressed in human breast cancer.

We report here that Rac1 is overexpressed in malignant breast tissue as compared to benign breast tissue. While we did not detect any activating mutation of Rac1 in the breast cancer tissues studied, we identified a new Rac1 isoform, Rac1b, with specific biochemical properties.

Results

Sequencing of Rac1 in breast cancer tissue identified single nucleotide polymorphisms in Rac1 and a new Rac1 isoform

The transforming ability of constitutively active mutants of Rac1 in cell culture raises the possibility that, in analogy to Ras, Rac1 might be mutated in breast cancer tissue *in vivo*. Seven normal mammary tissues and 10 breast cancer tissues (five with and five without disease recurrence) were analysed for mutations in the Rac1 coding sequence. After performing full length RT–PCR for Rac1 from each breast tissue specimen, the PCR products were subcloned and at least six clones from each tissue sequenced and compared to the wild-type (wt) Rac1 sequence. Most of the obtained sequences yielded wt Rac1. Eighteen different single mutations were identified in several clones but all except three mutations occurred in only one tissue, suggesting that they represent polymorphisms of the Rac1 gene in breast tissue. They were all submitted to the single nucleotide polymorphism (SNP) database (http://www.ncbi.nlm.nih.gov/SNP/) of the NCBI. Rac1 mutations occurred in three of five normal tissues, in one of two fibroadenomas, and six of the 10 breast cancer tissue specimens. There was an equal distribution of mutations between the group of patients with and without recurrence. Sequencing of Rac1 in eight different breast cancer cell lines identified one mutation each in the breast cancer cell lines MDA-231, ZR-75, and IDZ. These data were also submitted to the SNP database. The Rac1 mutation in the IDZ cell line occurred at codon 93 where two mutations were detected in a benign breast tissue. None of the mutations detected in breast tissue corresponded to constitutively activated mutations used in tissue culture (Qiu *et al*., 1995; Anand-Apte *et al*., 1997; Keely *et al*., 1997).

PCR amplification of Rac1 cDNA revealed a faint band above Rac1 in some of the breast tissue samples. After subcloning of the amplified PCR product, analysis of different Rac1 clones prior to sequencing showed, in addition to the wt Rac1 product (Figure 1a, lanes 1, 3 and 4) an amplified product of larger size (Figure 1a, lane 2). When this clone was sequenced, an insertion of 57 nucleotides in addition to the Rac1 wt sequence was found between nucleotides 225 and 226 of the Rac1 cDNA sequence (Didsbury *et al*., 1989) close to the switch region II (Figure 1b). This insert was named Rac1b as suggested (Jordan *et al*., 1999) and is located within the reading frame of the Rac1 sequence. It was confirmed by repeated sequencing in five normal and five malignant breast tissues. To confirm that no genomic Rac1 DNA sequence was amplified due to contamination of DNA when performing the PCR, we isolated mRNA from a breast tissue expressing both wt Rac1 and Rac1b. After reverse transcription and PCR amplification of Rac1, two products could be resolved by agarose gel

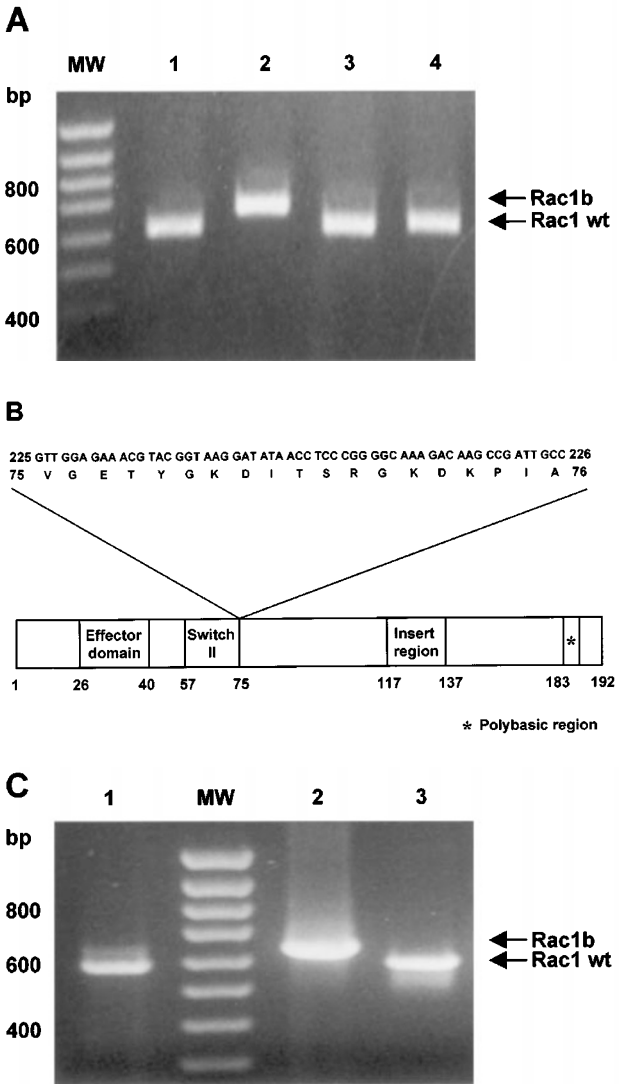

Figure 1 Identification of a 57 bp insert in Rac1 amplified from breast tissue. (**a**) After RT–PCR of Rac1 and subcloning into the TOPO-TA cloning vector the Rac1 fragment was excised and resolved on an agarose gel. After sequencing the products in lanes 1, 3, and 4 showed Rac1 wt, the product in lane 2 Rac1b. (**b**) Determination of the nucleotide sequence of the PCR product in lane 2 identifies a 57 bp-long insert between nucleotide 225 and 226 of the Rac1 wt sequence. (**c**) Total RNA was extracted from breast cancer tissue, the mRNA enriched with affinity columns, and an RT–PCR of Rac1 was performed (lane 1). After subcloning the PCR product in lane 1 and transformation, the two clones (lanes 2 and 3) were sequenced and resolved on an agarose gel. The clones in lanes 2 and 3 corresponded after sequencing to Rac1b and Rac1 wt, respectively

electrophoresis (Figure 1c, lane 1). Subcloning and sequencing revealed that the upper band corresponds to Rac1b and the lower band to Rac1 wt (Figure 1c, lanes 2 and 3). We assume that these bands represent two differentially spliced isoforms of Rac1 because the nucleotide sequences of the two cDNA species are identical except for the insert. To rule out that Rac1b is restricted to breast epithelium, we analysed Rac1b in normal gastric mucosal tissue and detected it there as well. We found Rac1b also in the Caco-2 (Bacher *et al*., 1992) colon cancer cell line but in none of the eight breast cancer cell lines analysed. A search through the GenBank Human Expressed Sequence Tag (EST) entries revealed an EST (aa526312) containing the insert in the context of a protein that had a 95% amino acid homology to Rac1. The insert was 100% identical to our reported insert. It was submitted but apparently not further characterized by the Cancer Genome Anatomy Project as part of a cDNA library made from microdissected tissue derived from prostatic intraepithelial neoplasia (Krizman *et al*., 1996).

Rac1b has accelerated GDP/GTP exchange

GTPases such as Rac1 regulate cellular functions by cycling through GDP and GTP states and interact with effectors in their active GTP-bound form. To analyse if the insert region affected GTP binding, affinity, or GTP/GDP exchange (activation), we compared *E. coli* derived (Figure 2a), purified GST-Rac1 with GST-Rac1b in their kinetic parameters. Exchange of [^{35}S]GTPγS from recombinant proteins was evaluated after separation of free and protein-bound GTPγS by filtration through nitrocellulose (Chuang *et al*., 1994). Within 1 min Rac1b exchanged about 70% of [^{35}S]GTPγS, while Rac1 exchanged only 30% (Figure 2b), suggesting that Rac1b exchanges bound GTPγS much faster than Rac1. Furthermore, analysis of the [^{35}S]GTPγS binding properties showed that Rac1b has a faster rate of nucleotide binding compared to Rac1 (data not shown). However, the intrinsic GTPase activity of Rac1 and Rac1b is similar, as shown in a GTP hydrolysis experiment (Figure 2c).

Rac1 mRNA and protein expression in benign and malignant breast tissue

Since we did not detect any mutations in the breast tissue specimens, which would suggest a constitutive activation of Rac1, we were interested in whether Rac1 is amplified or overexpressed in breast cancer tissue compared to normal breast tissue. At the time of surgery one piece of breast tissue was embedded in paraffin for immunohistochemistry and an adjacent piece snap frozen and stored in liquid nitrogen to be used for Western, Northern and Southern blot experiments. Rac1 expression was first examined by Western blotting with a monoclonal antibody to Rac1 from pulverized whole tissues (Figure 3a and data not shown). To quantify antigen expression, the blots were scanned and quantified by densitometry. The mean expression level of Rac1 (Figure 3b) was 2.7 times higher ($P<0.05$) in breast cancer tissue than in benign breast tissue. Despite the marked differences in disease recurrence and survival, both breast cancer subgroups had comparable levels of Rac1 protein. Similar results

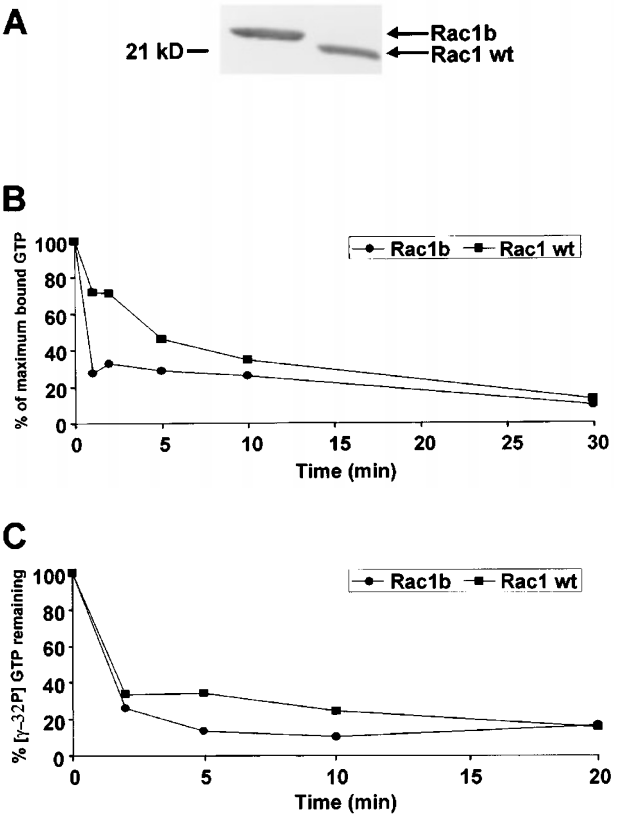

Figure 2 GTP exchange and hydrolysis of Rac1 and Rac1b. (**a**) Recombinant Rac1 and Rac1b were expressed in *E. coli* and resolved by SDS–PAGE. (**b**) Kinetics of nucleotide exchange after loading Rac1 and Rac1b with [^{35}S]GTPγS. Nucleotide exchange was initiated by the addition of GTPγS and bound [^{35}S]GTPγS analysed at the indicated time points. Values represent the mean of three independent experiments with similar results. (**c**) GTPase activity of Rac1 and Rac1b after loading with [γ^{32}P]GTP and measuring of hydrolysis at the indicated time points. Each point represents the average of two independent experiments

(data not shown) were obtained after analysing all the patient samples by Western blotting with a Rac1 polyclonal antibody (Quinn *et al*., 1993).

The finding that Rac1 protein is elevated in malignant over normal tissue prompted us to investigate the mechanisms responsible for the elevated expression of Rac1 in breast cancer. Total RNA was extracted from the breast tissue specimens used for Western blotting, and Northern blotting (Figure 3c) employing a Rac1 specific cDNA probe was performed as described before (Didsbury *et al*., 1989). Hybridization with Rac1 cDNA revealed two bands of 2.4 and 1.1 kB, respectively, present in 16 of 17 breast tissues; in one sample the RNA was degraded. The high level of Rac1 protein observed in Western blots was only partially a consequence of an abundant mRNA encoding Rac1. Comparing the average Rac1 RNA expression – after normalization for 18S rRNA between benign and malignant breast tissue showed on average a twofold induction (Figure 3d) for the 1.1 kb band. The 2.4 kb Rac1 band was expressed 1.4 times higher in malignant tissue. Additionally, some tissues displayed a relatively higher expression rate of the 2.4 kb Rac1 mRNA band compared to the 1.1 kB band. Southern blot hybridization on all 17 breast tissue specimens revealed no indication of Rac1 gene amplification in benign or malignant breast tissue (data not shown).

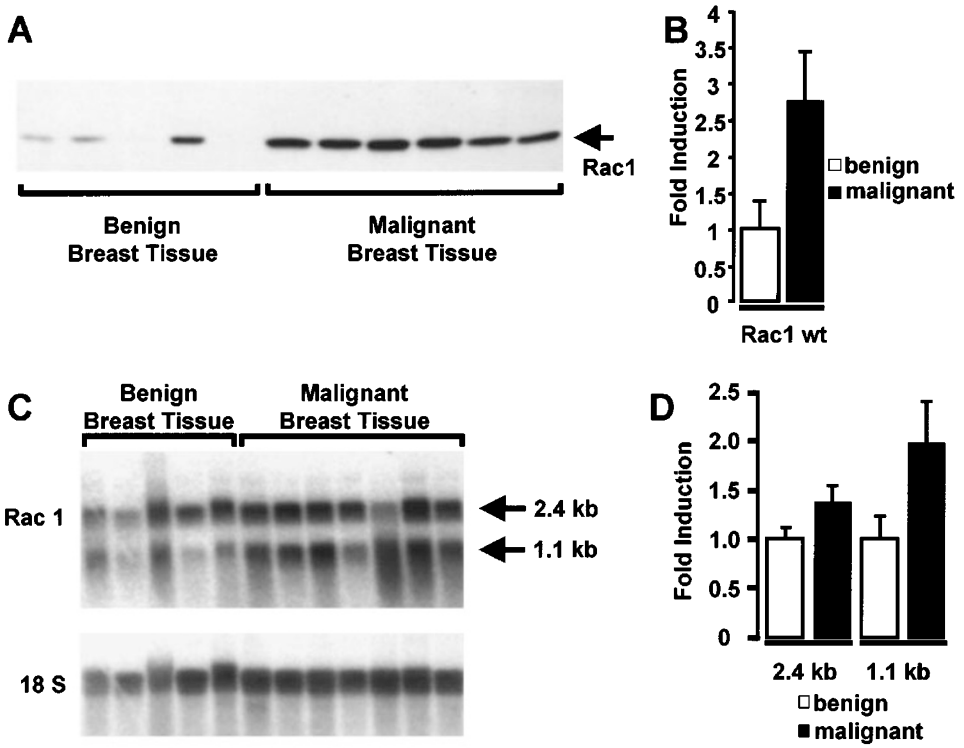

Figure 3 Rac1 protein and RNA is elevated in malignant over normal breast tissue. (**a**) Western blot. Breast tissue was extracted, normalized to equal protein (100 μg), and separated by a 10% SDS–PAGE. After transfer to nitrocellulose, the filter was incubated with a monoclonal antibody to Rac1. Reactive proteins were visualized by ECL. (**b**) The expression level of Rac1 from seven benign and 10 malignant breast tissues (**a** and data not shown) was determined by Western blotting and quantified using densitometric scanning. The induction rate of Rac1 in malignant breast tissue compared to benign breast tissue is expressed as fold induction. The data shown represent the average value and standard deviation. (**c**) Northern blot. RNA was extracted from breast tissue specimens, electrophoresed, and transferred to a nylon membrane. The filter was probed with cDNAs specific for the Rac1 and 18S-rRNA transcripts. (**d**) The expression level of Rac1 from seven benign and 10 malignant breast tissues (**c** and data not shown) was determined by Northern blotting and radioactivity quantified using a Phosphor Imager. The data shown represent the average value and standard deviation

Rac1 protein is highly expressed in proliferating epithelial breast cells and breast cancer

Western blot and Northern blot analysis of 17 breast tissue samples indicated that Rac1 expression is increased in breast cancer tissue compared to normal mammary tissue. However, these methods do not allow any conclusion regarding the cell type expressing the Rac1 protein. In order to substantiate our finding that Rac1 is overexpressed in breast cancer tissue, and investigate the role of Rac1 within the malignant transformation process of breast tissue immunohistochemical staining of Rac1 was performed in seven normal mammary tissues, 10 ductal carcinoma-in-situ, 20 invasive breast cancers and 10 corresponding lymph node metastases. Rac1 was stained weakly in epithelial cells of normal adult resting breast tissue (Figure 4a, open arrow), while there was no immunoreactivity detected in myoepithelial cells. The staining intensity of Rac1 was higher in fibrocystic disease or in epithelial hyperplasia (papillomatosis, Figure 4a, closed arrow) than in epithelial cells of a normal terminal-duct lobular unit, indicating that Rac1 protein is highly expressed in benign proliferating tissue. All 10 ductal carcinoma-in-situ displayed strong Rac1 expression (Figure 4b) in 60–100% of the tumor cells. All 20 primary breast carcinomas showed an intense brown granular staining, while adjacent normal breast tissue showed no or only weak staining. At least 50% of tumor cells stained positive in all breast cancer cases (Figure 4c,e) and all corresponding lymph nodes (Figure 4d,f). Semiquantitative assessment of Rac1 immunoreactivity showed no difference in staining intensity between primary lesion and lymph node metastasis (Figure 4c,d and e,f), whereas higher Rac1 expression was detected in carcinoma specimens compared to benign breast tissues. In breast carcinomas and lymph node metastases, two distinct staining patterns were observed: A cytoplasmic staining combined with a strong membrane staining (Figure 4c,d) or a diffuse cytoplasmic staining alone (Figure 4e,f). No distinct membrane staining was seen in proliferating as well as normal breast tissue. It is interesting to note that distinct membrane staining seemed to be more frequent in patients with relapse than in patients without. The median relapse-free survival in patients with strong plasma membrane staining was 35 months, compared to 69 months in patients without distinct membrane staining.

Discussion

Rac1 and Ras belong to the Ras superfamily of GTP-binding proteins which are key proteins in signal transduction pathways (Hunter, 1997). Characterization of the Ras oncogene elucidated that point mutations result in constitutive activation of Ras (Parada *et al*., 1982) and a high frequency of Ras mutations has been found in several human tumors, including breast cancer (Bos, 1989). In contrast to Ras,

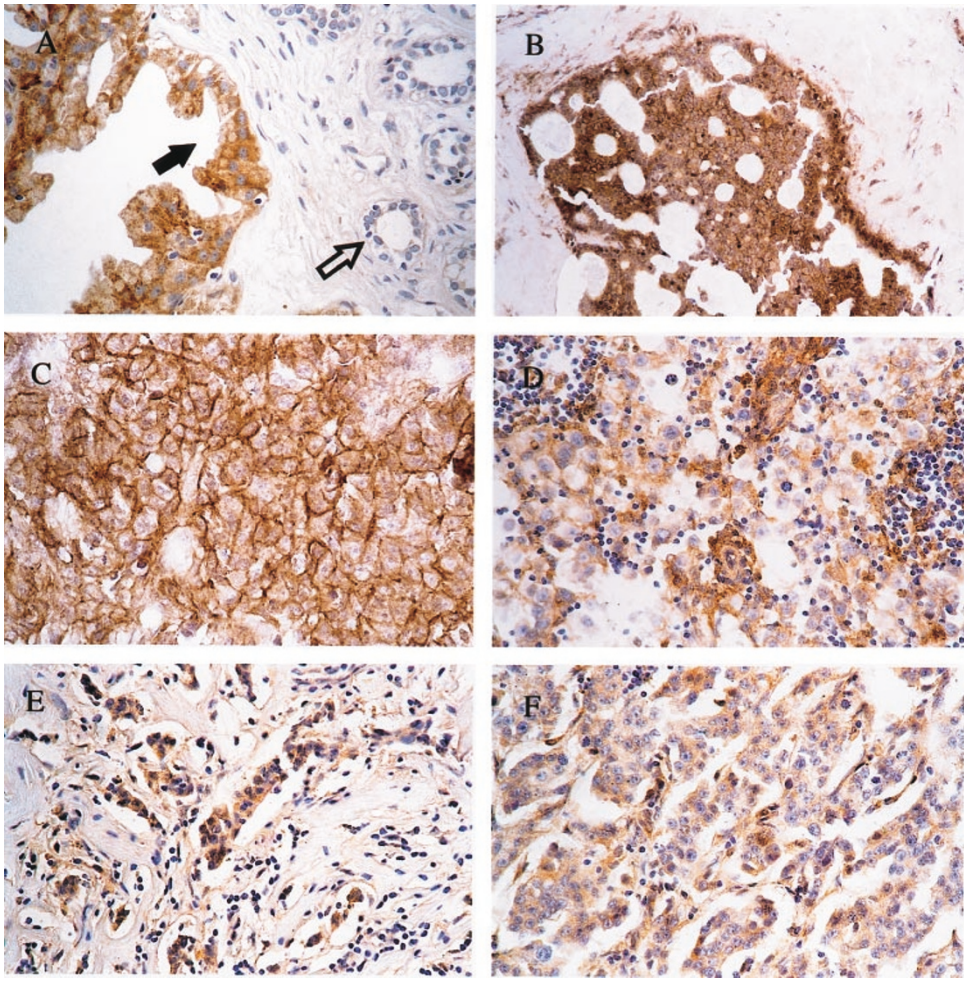

Figure 4 Immunohistochemical localization of Rac1 in breast tissue specimens. Tissue sections were incubated with a monoclonal antibody to Rac1 and staining visualized by the avidin-biotin peroxidase technique. A positive reaction shows a brown staining. (**a**) Fibrocystic disease with epithelial hyperplasia (closed arrow) shows strong Rac1 immunoreactivity, while normal, terminal-duct lobular units (open arrow) show weak Rac1 staining (1 : 200). (**b**) Ductal carcinoma-in-situ (1 : 100). (**c**) Invasive ductal carcinoma (1 : 400) and (**d**) corresponding lymph node with cytoplasmic and membrane staining of Rac1 (1 : 200). (**e**) Invasive ductal carcinoma (1 : 200) and (**f**) corresponding lymph node with only cytoplasmic staining of Rac1 (1 : 200)

the role of Rac1 in transformation has been analysed thus far only in cell lines (Qiu *et al*., 1995; Anand-Apte *et al*., 1997; Keely *et al*., 1997) by introducing constitutively activating mutations. To date, there has been no information whether these Rac1 mutations do indeed occur in human cancer tissue. By sequencing the Rac1 cDNA in normal and malignant human breast specimens as well as several breast cancer cell lines, we have identified a total of 18 different single mutations affecting the amino acid sequence at different positions throughout Rac1. The mutations identified are genetic variants of uncertain significance and probably SNPs. We did not detect any mutations indicative of a constitutive activation of Rac1 in human breast tissue and conclude that, at least for breast cancer, one has to be careful when transferring insights from the *in vitro* transforming abilities of mutated Rac1 to the *in vivo* situation. Nevertheless, constitutively activated mutants of Rac1 serve as useful tools for elucidating the activation sequence of different signal transduction proteins or cytoskeletal reorganization.

Although we did not find any single activating mutation in the Rac1 coding sequence *in vivo*, analysis of Rac1 at the RNA and protein level showed higher expression in malignant as compared to benign breast tissue, while we did not detect Rac1 gene amplification. These clinical data suggest that Rac1 expression in breast tissue is regulated either at the transcriptional level or through increased RNA stability. Studies on Rac1 regulation in tissue culture mainly considered GDP/GTP exchange and gave little attention to other regulatory levels, as exemplified by the fact that the 5′-regulatory region of Rac1 is still unknown. Therefore, in breast cancer tissue, in addition to the already characterized activation of Rac1 through GDP/GTP exchange, Rac1 expression is elevated and may contribute to increased activity.

When sequencing Rac1 in breast specimens, we identified an insertion of 19 codons within the reading frame between amino acid 75 and 76 close to switch region II. Analysis of the nucleotide sequence upstream and downstream of the insertion revealed consensus splicing sites. A literature search on alternatively spliced Ras-related GTP-binding proteins unveiled a protein called RagB (Schürmann *et al*., 1995) which has a 28-codon-long insertion between amino acid 77 and 78. Alignment of the potential 5′ and 3′ splice site of RagB and Rac1b showed that the insertion boundaries are homologous. We therefore assume that in analogy to RagB, Rac1 and Rac1b are derived from the same gene,

presumably by alternative mRNA splicing. While this work on breast cancer was under review, Jordan *et al.* (1999) published the presence of an identical Rac1 splice variant in human colon cancer although they did not report any biochemical properties of the new splice variant. Our experiments revealed that Rac1b is a functional GTPase. Comparison of the exchange activity of Rac1 and Rac1b shows that Rac1b has a very rapid nucleotide exchange. The high dissociation rate for nucleotides together with an intrinsic GTP hydrolysis rate similar to Rac1 suggests that the Rac1b protein is predominantly in the active, GTP-bound form. This was previously reported (Xu *et al.*, 1997; Lin *et al.*, 1997) for two Rho GTPases mutated in the Switch I region (Rac2 F28L, Cdc42Hs F28L) but not for any physiologically occurring GTPase. The biological role of Rac1b remains to be elucidated, particularly its role in signal transduction and cytoskeletal reorganization. Rac1b might play a role in colorectal tumor progression because Rac1b is increased in colorectal tumors compared to normal colonic mucosa (Jordan *et al.*, 1999). In breast cancer, we did not detect any difference in Rac1b expression between benign and malignant breast tissue, and preliminary experiments with cell lines have not shown any role of Rac1b in transformation (data not shown).

High Rac1 protein expression was detected immunohistochemically in epithelial hyperplasia, a benign proliferation of epithelial breast cells, in non-invasive ductal carcinoma-in-situ, and in invasive cancer. Because these are highly proliferating tissues, the data point toward a role of Rac1 in cell proliferation in accordance with previous results showing that overexpression of wt Rac1 in NIH3T3 cells induces a strong proliferative response (Burstein *et al.*, 1998) and that Rac1 stimulates cell cycle progression through G1 (Olson *et al.*, 1995). Because we see high expression of Rac1 already in ductal carcinoma-in-situ lesions, Rac1 in breast cancer is already present at the very beginning of the malignant transformation process and stays elevated throughout the metastatic process, as indicated by the high Rac1 expression detected in lymph node metastases. Distinct membrane staining for Rac1 was seen only in breast carcinoma cells but not in normal mammary tissue or proliferating lesions and was highly correlated with disease recurrence. Because active GTP-Rac1 is translocated to the plasma membrane (Fleming *et al.*, 1996), the membrane detection of Rac1 in human breast tumor tissue points toward active GTP-Rac1 in very malignant breast cancers. Therefore our *in vivo* analysis indicate two roles of Rac1 in human breast cancer: overexpression early in the course of transformation and activation of Rac1 in very aggressive breast cancers.

In summary, we found that Rac1 is overexpressed in proliferative breast disease, preinvasive and invasive breast carcinoma as well as lymph node metastases, but is located to the plasma membrane in tumor cells, only. In addition, we have identified a new Rac1 isoform, Rac1b, and characterized it as a functional GTPase.

Materials and methods

Patients

The study includes breast tissue specimens from 37 patients: five normal mammary tissues from patients receiving reduction mammoplasties (age: 21–32 years), two patients with fibroadenoma (30 and 46 years), 10 patients with ductal carcinoma-in-situ (encompassing van Nuys grades 1–3), and 20 patients with node-positive breast cancer (age: 33–79 years). In addition, one corresponding tumor-cell positive axillary lymph node was investigated for 10 cases with invasive carcinomas. For the Rac1 Western, Northern, and Southern blot analyses and Rac1 cDNA sequencing, a subset of fresh tissues consisting of the five normal mammary tissues, the two fibroadenomas, as well as 10 of the invasive carcinomas were used.

All breast cancer patients had undergone primary surgery and subsequent adjuvant systemic therapy at the Department of Obstetrics and Gynecology at the Technische Universität München. Primary therapy consisted either of modified radical mastectomy or wide tumor excision with axillary node dissection. In all patients treated with breast-conserving surgery, local radiation therapy was given. None of the patients had any clinical or radiological evidence of distant metastasis at the time of surgery. Clinical follow-up examinations were performed every 3–6 months for the first 5 years and then once a year. Median follow-up time was 60 months (range: 12–121 months). At analysis, 10 breast cancer patients had suffered disease recurrence while 10 patients had not recurred. Median overall survival was 37 months in patients with relapse, in contrast to 86 months in patients without relapse.

Immunohistochemistry

For immunohistochemical detection of the Rac1 protein, the avidin-biotin peroxidase technique was applied on formalin-fixed paraffin-embedded tissue sections (Schaller *et al.*, 1996) using the DAKO ChemMate Detection Kit DAB (DAKO, Hamburg, Germany). Briefly, paraffin sections were dewaxed, digested with 0.1% Protease (Sigma, Deisenhofen, Germany), and endogenous peroxidase activity blocked by 3% hydrogen peroxide in methanol for 30 min at RT. The monoclonal (UBI, Lake Placid, NY, USA) and polyclonal (Quinn *et al.*, 1993) antibody to Rac1 were incubated for 2 h at RT (10 μg/ml), respectively. Staining of the sections was assessed by a score considering mean staining intensity and the percentage of positive cells. At least five high power fields were examined.

Tissue extraction and Western blot analysis

Breast cancer tissue was obtained at surgery, stored on ice until frozen section diagnosis, snap frozen, and stored in liquid nitrogen. Breast tissue was pulverized by a Mikro-Dismembrator and resuspended in a buffer containing 0.02 M Tris-HCl (pH 8.5), 0.125 M NaCl and 1% Triton X-100 under gentle rotation at 4°C. The lysate was ultracentrifuged at 100 000 *g*, (45 min, 4°C) and an aliquot of the supernatant assayed for protein concentration. 100 μg of protein was electrophoresed in 12.5% SDS–PAGE under reducing condition. The resolved proteins were transferred to a nitrocellulose membrane (BA-S85, Schleicher & Schuell, Dassel, Germany) and the membrane subjected to a buffer containing 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl (pH 8.0), 0.25% gelatin (w/v), and 0.05% Triton X-100 and incubated sequentially with a 1 : 3000 dilution of the monoclonal antibody against human Rac1 followed by a horseradish peroxidase-conjugated anti-mouse IgG. Reactive proteins were visualized by ECL. The expression level of Rac1 was estimated using densitometric scanning with an Epson GT-9500 scanner and Scan Pack 3.0 software (Biometra, Göttingen, Germany).

Mutational analysis of Rac1 in breast cancer tissue

After manually homogenizing 100 mg of breast tissue which was stored in liquid nitrogen, 1 ml of TRIzol Reagent (GIBCO, Grand Island, NY, USA) was added and the viscous solution transferred to a microfuge tube. After addition of chloroform, the sample was centrifuged (12 000 *g*, 10 min, 4°C) and the aqueous phase precipitated by addition of 500 μl isopropanol and centrifuged again. Precipitated RNA was dissolved in RNase-free water and the concentration measured. After DNase-I digestion (GIBCO) reverse transcription was performed with random primer ($p(dN_6)$, Boehringer-Mannheim, Penzberg, Germany) and Superscript-II RNase H$^-$RT (GIBCO; 10 min, 25°C; 10 s, 30°C; 10 s, 35°C; 50 min, 42°C). For the PCR reaction, 2 μl of the resulting cDNA were incubated with 2.6 U Expand high fidelity polymerase (Boehringer-Mannheim), 200 μM dNTP, 2 mM $MgCl_2$ and 300 nM of each Rac1 primer (5′-primer: ATG CAG GCC ATC AAG TGT GTG GTG and 3′-primer: TTA CAA CAG CAG GCA TTT TCT CTT CC). The PCR reaction mixture was subjected to the following amplification process: 30 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 30 s. After amplification, reaction products were separated on 1.5% agarose gel and stained with ethidium bromide. The resulting PCR product was subcloned in the TOPO TA cloning vector (pCR 2.1, Invitrogen, Carlsbad, CA, USA) and transformed in competent *E. coli* using blue-white selection. White colonies were picked, cultured overnight, and plasmid DNA isolated. From each tissue at least six clones were sequenced using an ABI Prism 377 Genetic Analyzer (Perkin Elmer, Überlingen, Germany). Sequence analysis and comparison of the Rac1-clones were performed using the Wisconsin Package software (Version 9.0). All mutations were named using the convention of Baudet and Tsui (1993) with nucleotide number starting at the first transcribed base of Rac1 according to GenBank entry (M29870).

Extraction of mRNA from total RNA was performed with Oligotex columns (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The resulting mRNA was transcribed into cDNA, amplified, subcloned, and transformed. Plasmid DNA was extracted and sequenced.

Bacterial expression and purification of recombinant proteins

Rac1 and Rac1b were cloned into pGEX-2T (Invitrogen), transformed, and protein expressed after induction with IPTG. The recombinant GST (glutathione S-transferase) fusion proteins were purified on glutathione 4B beads and proteins recovered by cleavage with thrombin (Self and Hall, 1995).

Guanine nucleotide exchange

Recombinant Rac1 proteins (1.5 μg) were loaded with [^{35}S]GTPγS by incubation in 500 nM $MgCl_2$, 25 mM HEPES, 500 μM DTT, 50 μg/ml bovine serum albumin, 2.5 mM Tris, pH 8.0, in a total volume of 100 μl at 30°C. After 5 min 1 mM unlabeled GTPγS was added and the reaction stopped at the indicated time points, with 3 ml of cold stop solution, containing 9.15 mM $MgCl_2$. [^{35}S]GTPγS bound to protein was determined by filtration on nitrocellulose and liquid scintillation counting (Knaus *et al*., 1992). The optimal $MgCl_2$ concentration necessary for binding [^{35}S]GTPγS to recombinant Rac1 proteins was determined to be 500 nM.

Hydrolysis assay

GST-Rac1 and GST-Rac1b were incubated in 20 mM Tris-HCl, pH 7.6, 0.1 mM DTT, 25 mM NaCl, 4 mM EDTA, 0.16 mM $MgCl_2$, [γ-^{32}P]GTP, at 30°C for 10 min. Hydrolysis of GTP was initiated by the addition of 17 mM $MgCl_2$ and 1 mM GTP. Reactions were stopped with cold stop solution and binding of [γ-^{32}P]GTP to Rac1-proteins quantified by filtration and liquid scintillation counting (Knaus *et al*., 1992).

Northern and Southern blot

Total RNA was extracted from fresh breast tissue with TRIzol and 15 μg RNA electrophoresed in a 1% formaldehyde agarose gel and transferred to a positively charged nylon membrane by capillary action overnight (Lengyel *et al*., 1996). The Northern blot was probed at 65°C with a random primed, ^{32}P-labeled cDNA specific for the Rac1 mRNA (Didsbury *et al*., 1989). Loading efficiencies were checked by reprobing the blot with radioactive cDNA which hybridizes with the 18S rRNA. Signal intensities were quantified using a Molecular Dynamics 445 SI phosphor imager. The Southern blot was performed as described after digestion of genomic DNA with *Eco*RI (Lengyel *et al*., 1996).

Cell lines

Breast cancer cell lines MCF-7, MCF-7-LCC-1, and MCF-7-LCC-2 were provided by Dr N Brünner (Thompson *et al*., 1992), Finsen Laboratory, Copenhagen, Denmark. MDA-231, MDA-435, MDA-453, ZR 75 by Dr G Gallick, MD Anderson Cancer Center, Houston, TX, USA, IDZ by Dr Bühlmann, Pathology, University of Tübingen, Germany, and Caco-2 (Bacher *et al*., 1992) by Dr Prinz, Dept. Internal Medicine II, Technische Universität München.

Accession number

The sequence of Rac1b was submitted to GenBank (AF 136373), Rac1 polymorphisms to the SNP (NCBI) data base.

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