Analysis of c-*raf* Oncogene Expression in Gastrointestinal Tumor Cells

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A. Introduction

Oncogenes of retroviruses are responsible for the induction of tumors in animals and for the malignant transformation of cells in culture. They are derived from normal cellular proto-oncogenes. Comparison of differences between viral oncogenes and proto-oncogenes has indicated that they are never identical. In human tumors that are not induced by retroviruses, activated proto-oncogenes have been discovered which resemble the viral oncogenes in properties such as mutations, deletions, or amplified expression [1]. Activated oncogenes have been described in many different tumors or tumor cell lines [2]. Often, more than one oncogene seems to be activated, e.g., in HL60 promyelocytic leukemic cells *myc, myb* and *mil/raf* are amplified ([3] and unpublished observation). We are interested in the expression of the activated proto-oncogene c-*mil/raf* in human tumors. It is the homologue to the oncogene v-*mil* of the avian retrovirus MH2 [4]. While v-*mil* is expressed in virus-transformed cells as p100gag-*mil* fusion protein, the cellular homologue exhibits a molecular weight of 74 kD, p74hu-c-*raf* [5]. The v-*mil* protein is located predominantly in the cytoplasm and exhibits a virus-coded protein kinase activity in autophosphorylation reactions specific for the amino acids serine and threonine [6, 7]. It renders the cells independent of growth factors and therefore is involved in alteration of the signal transduction characteristic of many tumor cells.

In order to investigate growth parameters of pancreatic and other gastrointestinal tumors, we analyzed the expression of several proto-oncogenes at the RNA and protein levels. Here we performed immunoperoxidase analysis of cryosections of human pancreatic tissue specimens and cytological preparations of pancreatic, gastric, and colorectal cell lines using specific poly- and monoclonal antibodies against c-*mil/raf*. Additionally, we applied a c-*raf* cDNA probe for Northern blot hybridization of mRNA extracted from pancreatic tumor cell lines.

B. Results and Discussion

We used a DNA construct of v-*mil* and expressed a DNA fragment (TaqI/AccI) [8] in a prokaryotic expression vector pPLc24 as a MS2-v-*mil* fusion protein of 43 kD, p43 MS2-*mil* [9]. The protein expressed was used to raise monoclonal antibodies (mab) one of which recognizes a domain conserved among v-*mil* and hu-c-*raf* proteins (Fig. 1). This "mab" allows detection of the p100gag-*mil* protein by indirect immunoprecipitation from metabolically labeled cells (Fig. 2) and the p74hu-c-*raf* protein. A carboxy-terminal synthetic peptide was also used to raise polyvalent antibodies ("C-term ab") against the p100gag-*mil* protein (Fig. 2). It also recognizes the p74hu-c-*raf* protein. Using Western blot techniques (Fig. 3), p74hu-c-*raf* protein was

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detected in the two pancreatic tumor cell lines Capan-1 and QGP-1 by both, "mab" and "C-term ab". The latter recognized a 200-kD protein in addition, which may be due to a cross-reactivity of the polyclonal IgG. In rodent F12C2 cells "p45-50 *raf*" protein(s), representing truncated c-*raf*, were clearly stained

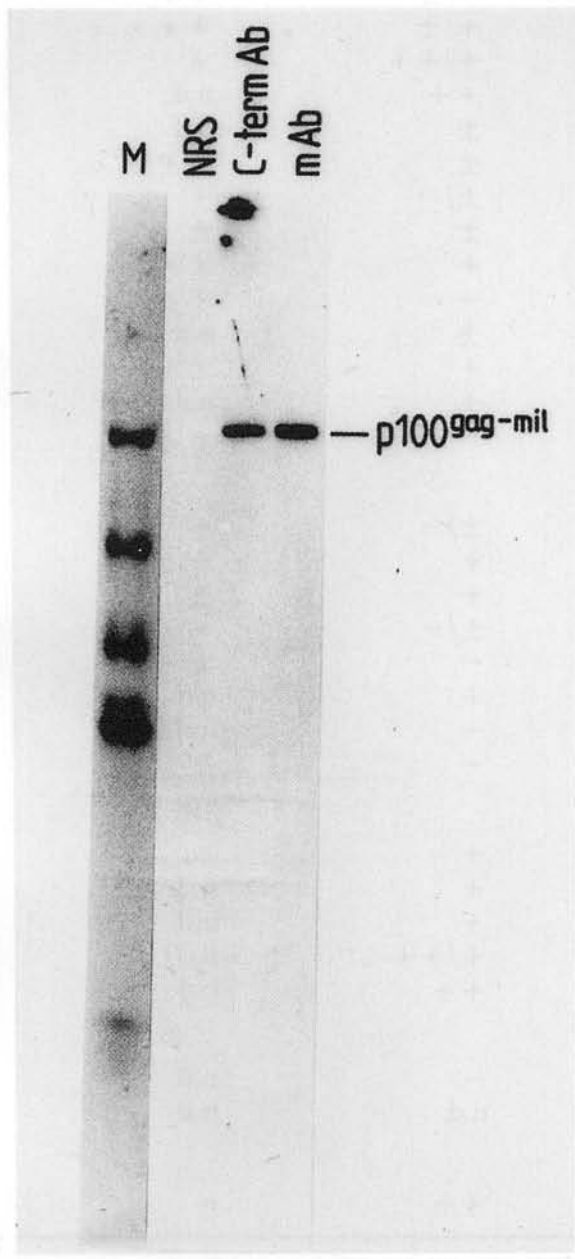

Fig. 1. Antigen specificity of applied antibodies. ^{35}S-radiolabeled MH2-transformed fibroblasts were lysed and immunoprecipitated with normal rabbit serum (NRS), with polyclonal rabbit IgG (C-term ab), or with mouse monoclonal antibody (mab; compare legend of Table 1), run on SDS-polyacrylamide gels, and autoradiographed. M, Molecular weight standards

by both antibodies, "mab" showing two prominent bands in comparison with four bands recognized by "C-term ab". Competition experiments using the two antibodies and their purified respective antigens confirmed the specificity of the two antibodies in immunohistological analysis (data not shown).

With respect to pancreatic tumor cell lines (Table 1) most of them stained positively in the cytoplasm: 8 times clearly positive, 6 times weakly positive, 3 times negative, with both antibodies showing similar staining intensities in most cases ($n=17$). While all colorectal cell lines ($n=5$) were found to be clearly positively stained, the gastric cancer cell lines ($n=8$) reacted less intensely (3 times clearly positive, 2 times weakly positive, 3 times negative) (Table 1). The immunocytological data were in good agreement with the Northern blot results (Table 1, Fig. 4). Only five of 20 samples showed positive hybridization signals in Northern blots, while the corresponding proteins showed only weak immunocytological reactivity if any. Whether this represents different sensitivities of the applied methods or is due to post-transcriptional regulation remains to be shown. In primary ductal adenocarcinomas of the pancreas ($n=18$), fifteen specimens showed positive reactions (mostly of the ductal cells) when tested with the *mil/raf* specific mab in immunohistochemical analyses (Table 2). Interestingly, normal ($n=9$) and chronically inflamed ($n=3$) pancreatic tissues revealed a positive acinar cell reactivity in addition to some ductal cell staining in nearly all samples. Staining was confined to the cytoplasm in most cases, and was particularly prominent in mitotic cells of the QGP-1 pancreatic tumor cell line.

Some cases of pancreatic tumor cell lines, as well as ductal epithelial cells of exocrine pancreatic tumors, revealed a nuclear localization of immunoreactive protein as detected by mab.

These results suggest a possible function of c-*raf* encoded protein expression in proliferating as well as differentiated

Table 1. Analysis of c-*mil/raf* oncogene expression in cell lines

Cell line	Immunoperoxidase staining		Northern blot analysis
	C-term Ab	mAb	c-*raf*-1
Pancreatic cancer			
Capan-2	−	−	−
Colo-357	+	+	++
ASPC-I	+	+	++
SW-850	−/±	+/±	++
SW-979	+	+/±	++
QGP-1	+	+/++	+
Panc-89	±	++	n.d.
Panc-Tu I	−/±	±	n.d.
Panc-Tu II	−/+	±	++
HPAF	±/−	±/−	+
BXPC-3	±	±	±
Capan-1	+	+	+++
PT-45-P1	n.d.	−	++
A-818 (−1)	+/±	±	n.d.
A-818(−4)	+	+	±
A-818(−5)	+	+	n.d.
A-818(−7)	+/−	−	−
Gastric cancer			
MKN-7	±/−	±/−	+
MKN-28	n.d.	+	+/±
MKN-45	+	+	±
MKN-74	−	±/−	+
Kawasaki	±/−	−	±/−
Okajima	±	+	n.d.
SCH-1013	n.d.	−	n.d.
MS1-P18	±/−	−	±
Colonic cancer			
WIDR	+	+	+
SW-1116	+/±	+	n.d.
HT-29	+	+	n.d.
E-5583	+/++	+/++	n.d.
Colo-320-HSR	+/++	++	n.d.
Normal fibroblasts			
F2	−	−	n.d.
F14	n.d.	n.d.	n.d.
Transformed fibroblasts			
F12C2	++	++	+

Immunoperoxidase staining was performed using the "mab" or "C-term ab". As a control another monoclonal antibody directed against a v-*mil*-specific determinant, was found to be negative in all samples tested ($n=32$). Expression of c-*raf* mRNA was studied by Northern blot analysis using a human c-*raf*-specific c-DNA probe (Amersham/Buchler). Relative intensities of reactions are given as "−" (negative), "+/−" (weak), "+ or ++" (moderate or strong), "n.d." (not done), "+++" indicates a strong positivity in Northern blot analysis with an additional 6.2 kb transcript, "/" indicates heterogeneity or variability of results. The pancreatic cancer cell lines tested include five primary cell lines, which have been recently established in our lab. Besides gastric and colonic tumor cell lines normal human fibroblasts as well as transformed rodent fibroblasts were tested.

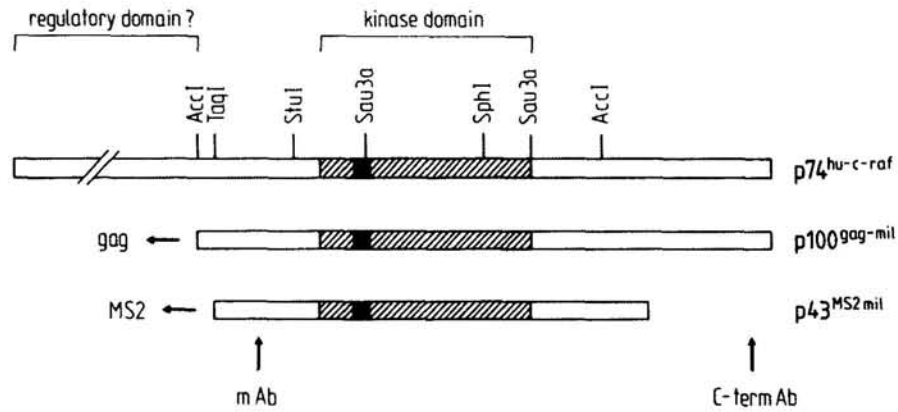

Fig. 2. Schematic epitope localization of applied antibodies. Comparison of p74-human-c-*raf* structure with p100gag-*mil* from MH2 virus and with recombinant bacterial fusion protein p43MS2-*mil*. *Arrows* indicate epitope localization of mouse monoclonal antibody (mAb) and of rabbit polyclonal IgG (C-term Ab). The *black box* represents the ATP-binding site

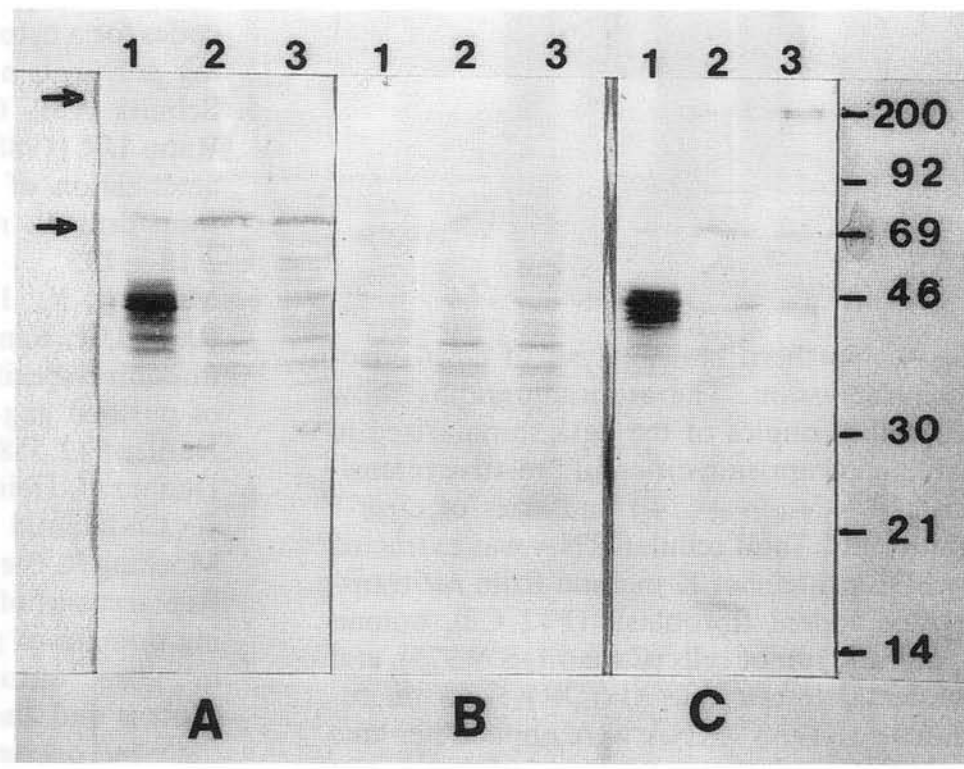

Fig. 3. Western blot analysis of c-*mil/raf* encoded protein. Cells were solubilized in 0.1% Triton-X-100 buffer and extracts were clarified by 100000 × g centrifugation. Aliquots of 50 µl were run on 12.5% SDS-polyacrylamide-gels and blotted on nitrocellulose using standard procedures. Lane 1, F12 C2; lane 2, Capan 1; lane 3, QGP-1. Overlay of panel *A* with "mab", *B* with control IgG1, *C* with C-term "ab". Molecular weight markers are given in kD. The *lower arrow* indicates p74hu-c-*raf*, the *upper arrow* points to a 200-kD protein – doublet detected in QGP-1 cells by C-term "ab"

Table 2. Immunoperoxidase studies of c-*mil/raf* oncogene expression in pancreatic tissue.

Tissue specimen	Number, score of staining intensity
Ductal adenocarcinoma	15 × +, 3 × – (mainly ductal cells)
Chronic pancreatitis	3 × + (acinar and ductal cells)
Normal pancreas	8 × +, 1 × – (acinar and ductal cells)
Endocrine tumors	3 × +, 1 × – (acinar and tumor cells)

Cryosections were incubated with "mab" (compare legend of Table 1). A negative control antibody showed only some background staining in a few cases (data not shown).

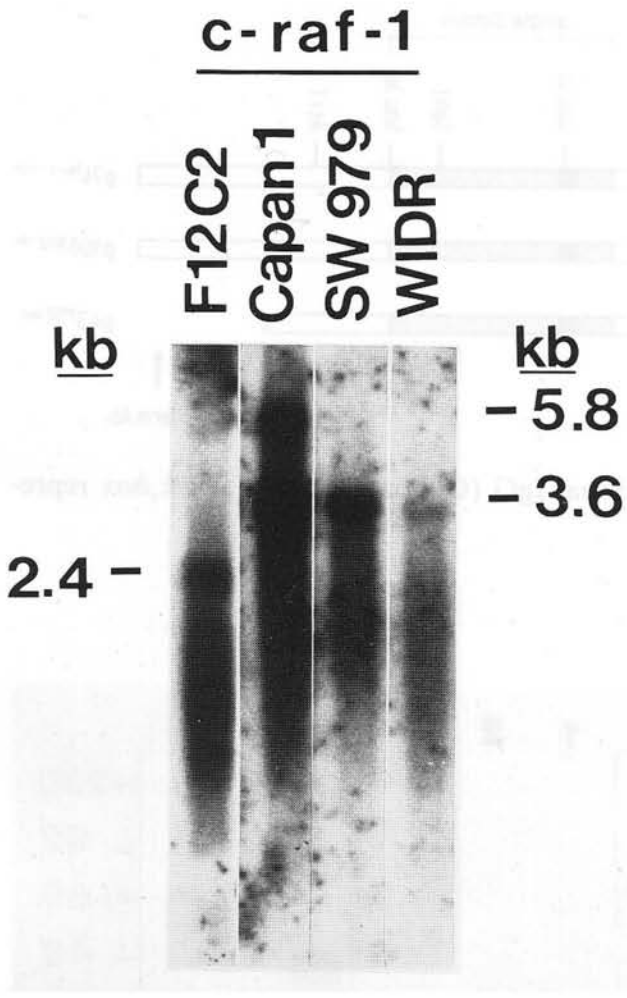

Fig. 4. Northern blot analysis of c-*raf* oncogene expression. The autoradiographs show selected examples of the data summarized in Table 1, comprising different "relative intensities" (+++, ++, +) and sizes of c-*raf*-1 transcripts. Total cellular RNA was extracted by SDS/proteinase K method from *raf*-transformed rodent fibroblasts (F12 C2), human pancreatic tumor cells (Capan 1, SW 979), and colorectal tumor cells (WiDR). Two micrograms polyl $(A)^+$-RNA were applied per lane and a ^{32}P-labeled, c-*raf*-1-specific DNA probe (Amersham/Buchler) was used for hybridization. Molecular weights of the transcripts were determined according to an RNA ladder (BRL)

pancreatic cells; this parallels recent findings reported for the *ras* protein [10]. The prominent immunohistochemical staining of mitotic cells and the positivity of the nuclei in some pancreatic tumor cell lines clearly present further questions on the biological role of c-*mil*/*raf* in the signal transduction pathway and require additional investigations.

References

1. Bishop JM (1985) Viral oncogenes. Cell 42:23–38
2. Moelling K, Heimann B, Bading H, Häder M, Bepler G, Havemann K, Beutler C (1987) Modulation of liver cell expression. MTP/Kluwer, Lancaster, pp 275–285
3. Collins SJ, Ruscetti FW, Galagher RE, Gallo RC (1978) Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfooxide and other polar compounds. Proc Natl Acad Sci USA 75:2458–2462
4. Bunte T, Greiser-Wilke T, Moelling K (1983) The transforming protein of the MC29-related virus CMII is a nuclear DNA-binding protein whereas MH2 codes for a cytoplasmic RNA-DNA binding polyprotein. EMBO J 2:1087–1092
5. Schultz AM, Copeland T, Oroszlan S, Rapp UR (1988) Identification and characterization of c-*raf* phosphoproteins in transformed murine cells. Oncogene 2:187–193
6. Moelling K, Heimann B, Beimling P, Rapp UR, Sander T (1984) Serine- and threonine-specific proteinkinase activities of purified gag-*mil* and gag-*raf* proteins. Nature 312:558–561
7. Denhez F, Heimann B, d'Auriol L, Graf T, Coquillaud M, Coll J, Galibert F, Moelling K, Stehelin D, Ghysdael J (1988) Replacement of Lys 622 in the ATP binding domain of p100gag-*mil* abolishes the in vitro autophosphorylation of the protein and the biological properties of the v-*mil* oncogene. EMBO J 7:541–546
8. Kan NC, Flordellis CS, Mark GE, Duesberg PH, Papas TS (1984) Nucleotide sequence of the avian carcinoma virus MH2: Two potential onc genes, one related to avian virus Mc29 and the other related to murine sarcoma virus 3611. Proc Natl Acad Sci USA 81:3000–3004
9. Moelling K, Pfaff E, Beug H, Beimling P, Bunte T, Schaller HE, Graf T (1985) DNA-binding activity is associated with purified *myb* proteins from AMV and E26 viruses and is temperature-sensitive for E26 ts mutants. Cell 40:983–999
10. Furth ME, Aldrich TH, Cordon-Cardo C (1987) Expression of *ras* proto-oncogene proteins in normal human tissues. Oncogene 1:47–58