that this protein might colocalize to some CRF pathways and modulate the neural actions of this neuropeptide. □

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Raf-1 protein kinase is required for growth of induced NIH/3T3 cells

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MANY growth factors regulate the cytoplasmic Raf-1 protein kinase[1–10], consistent with its having a central role in transduction of growth signals. The kinase is ubiquitously expressed[11] and can promote proliferation[12], presumably in a manner dependent on growth-factor receptors and membrane-associated oncogenes[13–15]. We have now examined the dependence of serum- and TPA (12-*O*-tetradecanoylphorbol-13-acetate)-regulated NIH/3T3 cell growth on RAF-1 kinase to determine whether Raf-1 is essential for receptor signalling. We inhibited Raf-1 function by expressing c-*raf*-1 antisense RNA or kinase-defective c-*raf*-1 mutants. Antisense RNA for c-*raf*-1 interferes with proliferation of normal NIH/3T3 cells and reverts *raf*-transformed cells. In revertant cells, DNA replication induced by serum or TPA was eliminated or reduced proportionately to the reduction in Raf protein levels. Expression of a kinase-defective Raf-1 mutant (craf301) or a regulatory domain fragment (HCR) inhibited serum-induced NIH/3T3-cell proliferation and *raf* transformation even more efficiently. Inhibition by antisense RNA or craf301 blocked proliferation and transformation by Ki- and Ha-*ras* oncogenes. We conclude that *raf* functions as an essential signal transducer downstream of serum growth factor receptors, protein kinase C and *ras*.

Portions of c-*raf*-1 cDNAs were expressed in sense and antisense orientation using the pMNC vector (Fig. 1). After transfection into NIH/3T3 cells the number of neomycin-resistant colonies was scored. Antisense constructs yielded roughly half the number of colonies as did the corresponding sense construct or the vector control, indicating that *raf* antisense RNA interferes with viability and/or proliferation. As NIH/3T3 cells express no B-raf and 10-fold less A-raf than Raf-1 (ref. 11), we ascribe the effect to interference with Raf-1. Antisense colonies were generally smaller and grew slower than sense or vector control colonies. Ten out of ten antisense colonies showed barely detectable levels of Raf-1 protein, whereas levels in sense control clones were unchanged (data not shown). An alternative approach to Raf-1 inhibition used inactive mutants[13,16]. A truncated Raf-1 protein (HCR) corresponding to conserved region 1 reduced colony numbers fourfold. A kinase-defective Raf-1 mutant protein, craf301 (plasmid p301-1), was even more efficient, decreasing colony yield about sevenfold[16]. The surviving colonies from these experiments could not be maintained as stable cell lines. We therefore turned to *raf*-transformed cell-lines where morphological reversion and inhibition of proliferation could be studied. p301 constructs were transfected into 208-F12 fibroblasts which overexpress a transforming mouse Raf-1 protein[17]. p301-2 caused partial or complete reversion of the transformed phenotype in approximately half the transfectants. Reversion correlated with loss of anchorage-independent growth. Again, p301-1 was more efficient than p301-2 (Fig. 2*a*). These clones were unstable, but cell-lines sufficiently stable for biochemical analysis were obtained after

TABLE 1 *raf*-inhibition blocks *ras*-mediated proliferation and transformation

a) v-Ki-*ras* cell transfection

Plasmids	Yield of neo^r colonies	Morphology of neo^r colonies: flat	intermediate	transformed
pMNC*	100 ± 0%	0 ± 0%	0 ± 1%	100 ± 1%
pMNC301-2†	61 ± 8%	2 ± 1%	15 ± 7%	83 ± 7%
pMNC301-1‡	30 ± 7%	15 ± 3%	15 ± 5%	70 ± 6%

b) NIH/3T3 co-transfection with v-Ha-*ras* (pSV2neo/ras) and p301

Plasmids (ratio 1:1)	Inhibition§	Morphology of neo^r colonies: flat	intermediate	transformed
ras + pMNC*	0 ± 3%	27 ± 3%	17 ± 5%	56 ± 3%
ras + p301-2†	53 ± 4%	28 ± 1%	46 ± 9%	26 ± 7%
ras + p301-1‡	61 ± 3%	46 ± 4%	32 ± 7%	22 ± 4%
Plasmids (ratio 1:4)				
ras + pMNC*	0 ± 1%	33 ± 4%	23 ± 3%	44 ± 3%
ras + p301-2†	61 ± 5%	48 ± 5%	35 ± 7%	17 ± 4%
ras + p301-1‡	84 ± 4%	67 ± 4%	25 ± 6%	7 ± 3%

Cells were transfected and G418-resistant (400 μg ml^{-1}) colonies were morphologically examined according to the criteria described in Fig. 2. Percentages are calculated for two experiments with ≥200 (*a*) or ≥400 (*b*) colonies per transfection.
* Vector control.
† Antisense orientation.
‡ Sense orientation.
§ The efficiency of inhibition of *ras* transformation is given as percentage reduction in the number of transformed colonies.

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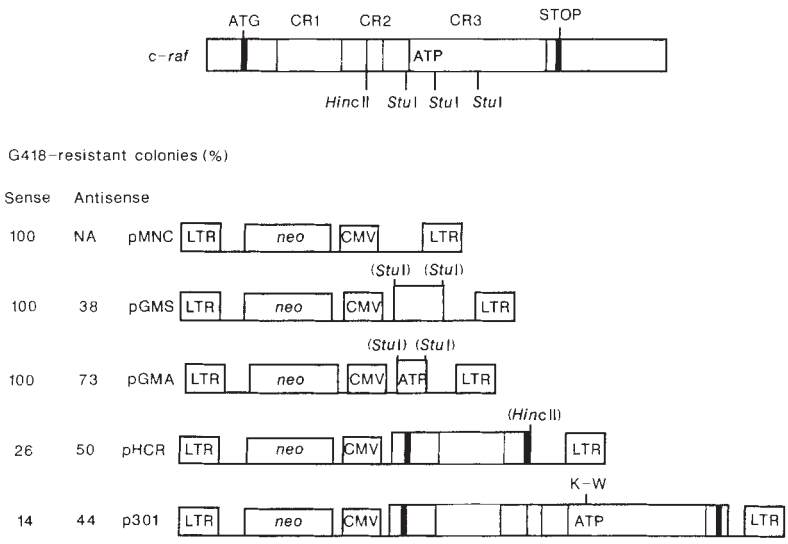
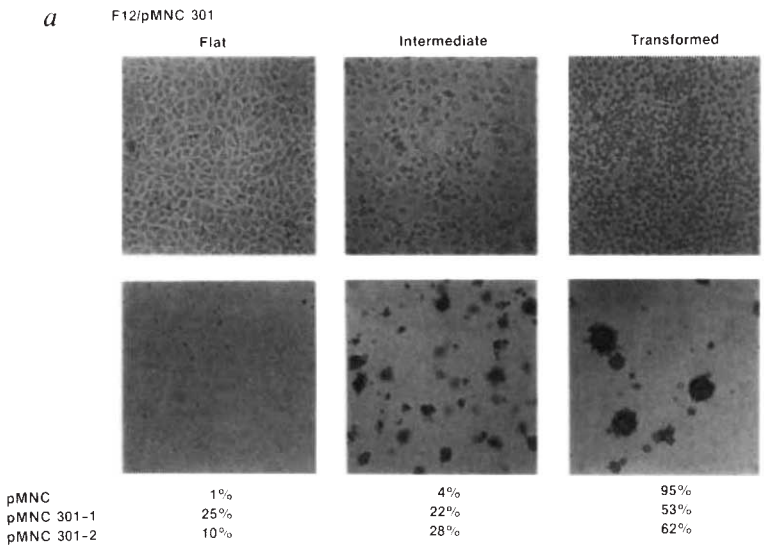
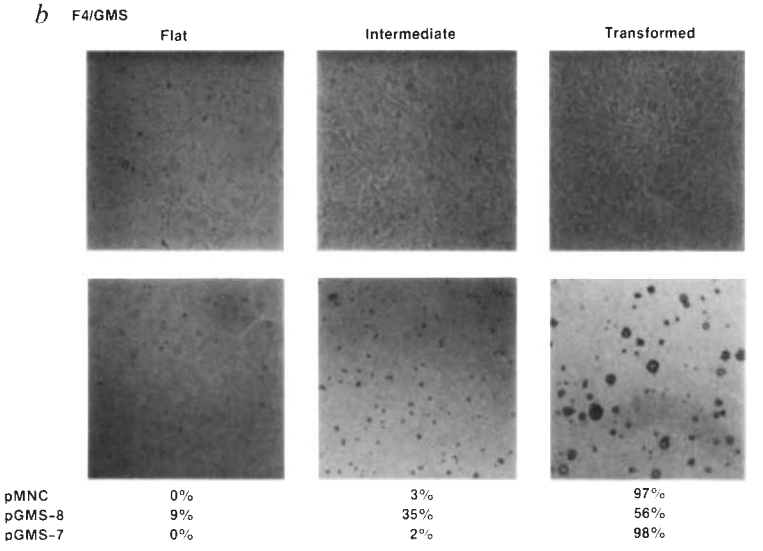


FIG. 1 Schematic diagram of murine and human c-*raf*-1 cDNAs and expression plasmids used. GMA and GMS contain *Stu*I restriction fragments of the mouse c-*raf*-1 cDNA (G. H., unpublished data), HCR an N-terminal *Hin*cII fragment of the human cDNA. p301 consists of all the coding sequence of a mutant human c-*raf*-1 cDNA. The lysine(375) to tryptophan (K→W) mutation in the ATP-binding site is indicated[16]. Restriction fragments were cloned in both sense and antisense orientation. NIH/3T3 cells were transfected with sense and antisense plasmids, and with the pMNC vector as control. G418-resistant (400 $\mu g\ ml^{-1}$) colonies containing more than 50 cells were counted after three weeks. The pMNC vector served as internal standard. The experiment was repeated three times (twice for HCR) with different batches of plasmid preparations. Variations between experiments were in the order of 10% but did not affect the ratios between the different constructs shown. CR1–3, conserved regions; ATP, ATP-binding domain; LTR, mouse Moloney virus long terminal repeat; NEO, neomycin-resistance gene; CMV, cytomegalovirus immediate early promoter.

METHODS. pMNC digested with *Xho*I and *Bam*HI was blunt ended with T4 DNA polymerase. The mouse and human cDNAs were cut with *Stu*I or *Hin*cII, respectively, and appropriate sized fragments were ligated with the pMNC vector. GMA contains residues 1254–1426 and GMS 1427–1697 of the mouse cDNA, HCR 1–903 of the human c-*raf*-1 cDNA[25]. The translation termination codon for HCR sense is provided by vector sequences resulting in the addition of nine amino acids. To construct p301-1 (sense orientation), an *Eco*RI–*Xba*I fragment of p628 (ref. 25) encompassing the coding sequence of a human c-*raf*-1 cDNA was cloned into BluescriptKS (Stratagene). Lysine(375) was changed to tryptophan by site-directed mutagenesis resulting in the creation of a unique *Bam*HI site. This cDNA was transferred into the *Sac*I–*Xho*I sites of pSVL (Pharmacia), then cloned into the *Xho*I–*Bam*HI sites of pMNC as an *Xho*I–*Bam*HI (partial digest) fragment. The corresponding antisense plasmid, p301-2, was generated by cloning the blunt-ended c-*raf* 301 *Eco*RI–*Xba*I fragment into blunt-ended pMNC.





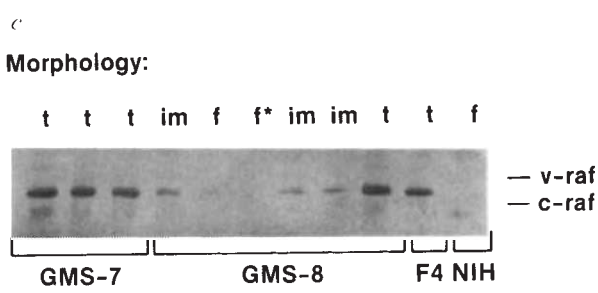


FIG. 2 Morphological reversion of *raf*-transformed cells by transfection with *raf* antisense and mutant constructs. *a*, p48 *raf*-transformed 208/F12 (ref. 17) or *b*, v-*raf*-transformed F4 (ref. 18) fibroblasts were transfected with plasmids p301-1 (sense) and 301-2 (antisense) or GMS-7 (sense) and GMS-8 (antisense), respectively, as well as with the pMNC vector. Monolayer growth with minor irregularities and a decreased ability to form soft agar colonies was categorized as partial reversion. Flat clones showed no areas of overgrowth and did not form colonies in soft agar. *c*, A representative analysis of Raf protein expression in individual cell clones. t, Transformed; im, intermediate; f, flat (clone GMS-8/2); f* (clone GMS-8/3).

METHODS. Cells were lysed in TBST (150 mM NaCl, 20 mM Tris HCl, pH7.5, 2 mM EDTA, 1% Triton X-100, 1 mM PMSF). Lysates were adjusted to equal protein concentrations (Biorad protein assay kit). Raf proteins were precipitated with the monoclonal antibody PBB1 and analysed by western blotting with the polyclonal serum #137 as described previously[26].

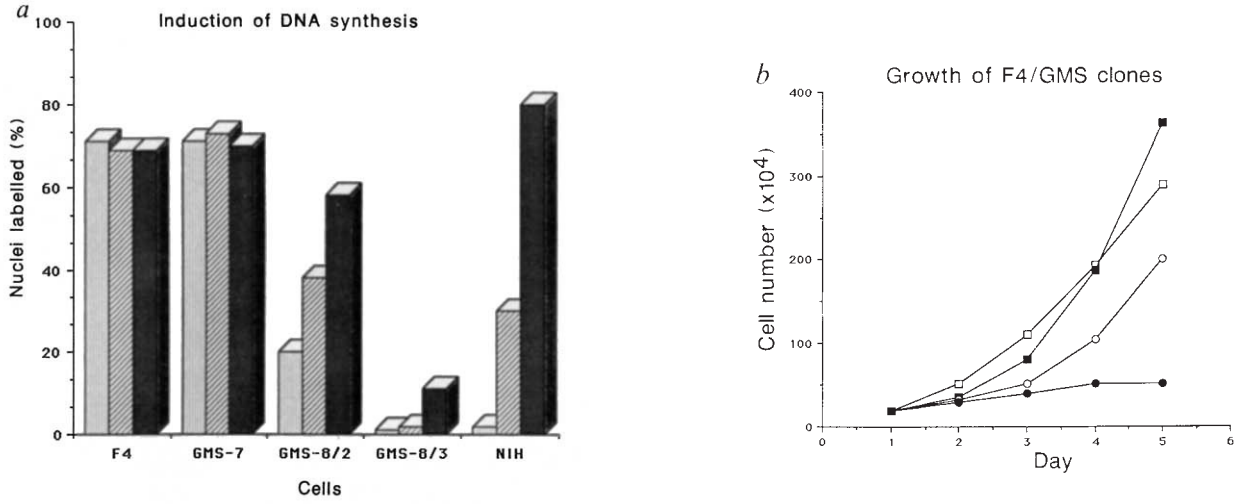


FIG. 3 Mitogen responsiveness and proliferative capacity of Raf-depleted cells. *a*, DNA synthesis induced by serum or TPA in serum-starved cells is depicted as the number of nuclei incorporating ^{3}H-thymidine. *b*, Long term growth curves GMS-7 is a pool of 10 clones transfected with sense DNA, GMS-8/2 and GMS-8/3 are flat clones reverted with antisense DNA. *a*, ▨, starved cells; ▨, TPA-induced cells; ■, SERUM-induced cells. *b*, □, F4; ■, GMS-7b; ○, GMS-8/2; ●, GMS-8/3.
METHODS. 10^4 cells were plated on cover slips and serum-starved for 24 h before incubation with 20% foetal calf serum (Gibco) or 100 ng ml^{-1} TPA (Sigma). 14 h after addition of mitogens, cells were labelled with 1 μCi ml^{-1} ^{3}H-thymidine for 5 h. Cells were counter-stained with Giemsa and labelled nuclei were counted. For long-term growth curves, 10^5 cells were seeded in six-well plates in DMEM medium supplemented with 10% FCS. Each day, one well was trypsinized and counted with a Coulter cell counter. All determinations were performed in triplicate.

pGMS transfection of v-*raf* transformed cells, F4 (ref. 18). Neither pMNC nor the control plasmid GMS-7 was effective, whereas the antisense construct, GMS-8, completely or partially reverted F4 (Fig. 2*b*). Reduction of *raf* mRNA (data not shown) and protein levels correlated with the extent of reversion (Fig. 2*c*). In one clone, GMS-8/3 (marked f* in Fig. 2*c*), *raf* protein expression was undetectable. These cells grew extremely slowly, arresting at 50–60% confluency, and eventually died.

To measure the effects of *raf*-protein depletion on the mitogen response, we determined the ability of serum and TPA to induce DNA synthesis in serum-starved cells (Fig. 3*a*). F4 and GMS-7 cells synthesize DNA independently of mitogens. Constitutive DNA synthesis was diminished in GMS-8/2, which retained an inducible response similar to NIH/3T3 cells. GMS-8/3 was completely blocked in constitutive and TPA-inducible DNA replication. Serum-stimulation of GMS-8/3 was reduced seven-fold, and long-term growth was also severely diminished (Fig. 3*b*).

There is ample evidence for control by *ras* of the signal flow from membrane receptor systems[14,15,19–22]. We thus transfected v-Ki-*ras*-transformed NIH/3T3 cells with the p301 plasmids (Table 1*a*). p301-1 and p301-2 reduced neomycin (neo)-resistant colony yield to a similar degree as in NIH/3T3 cells (Fig. 1), suggesting that Raf-1 is required for proliferation of *ras*-transformed cells. Morphological reversion of established *ras*-transformed cells was less efficient than of *raf*-transformed cells (Fig. 2). To test the effect of *raf*-inhibition on the initiation of *ras*-transformation, a constant amount of v-Ha-*ras* (pSV2neo/*ras*, (ref. 24) plasmid was co-transfected with an equal or four-molar amount of the p301 vectors (Table 1*b*). Although the neomycin resistance of pMNC-based plasmids accounted for a background of flat neo^r colonies that presumably did not express pSV2neo/*ras*, transfection with p301 vectors markedly increased the number of morphological revertants at the expense of transformed colonies. The inhibition was dose-dependent and almost complete at four-molar excess of p301-1.

We conclude that in NIH/3T3 cells Raf-1 kinase functions downstream of membrane receptors and *ras* proteins and is essential for growth-induction by serum factors and protein kinase C. Membrane receptor systems can now be examined individually for Raf-1-dependence by inhibition with the blocking constructs described here. Furthermore, the proposed position of *raf* in the communication pathway between cell membrane and nucleus makes *raf* an attractive target for the design of novel antiproliferative agents, especially as our data show that *raf* inhibition is dominant over transformation by *ras* and, by implication, by other non-nuclear oncogenes. □

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