Raf Function Is Required for Proliferation of NIH|3T3 Cells and Transformation by Nonnuclear Oncogenes

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Studies of oncogenes have established that their cellular homologs are part of a kinase cascade mechanism that regulates the translation of exogenous growth factor stimulation into a mitogenic gene response [reviewed in 1]. The cellular homolog of the v-*raf* oncogene [2], c-*raf*-1 [3], encodes a mitogen-regulated serine/threonine-specific protein kinase, whose specific activity is enhanced by a large number of growth factors [4–13]. Moreover, treatment of NIH/3T3 fibroblasts with platelet-derived growth factor (PDGF) or 12-*O*-tetradecanoylphorbol-13-acetate (TPA) causes translocation of a fraction of Raf-1 from the cytosol to the nucleus [12]. A similar fraction of v-Raf is found associated with the nucleus also in the absence of stimulation (W. B. Anderson and U. R. Rapp, unpublished results). These observations led to the speculation that Raf-1 serves as a shuttle enzyme which converts peripheral signals into a transcriptional response that results in mitogenesis [14]. Consequently, a key step in transformation can be envisioned to uncouple Raf-1 kinase from mitogen regulation, either through constitutive stimulation by upstream signal transducers or mutational activation of *raf* itself. This hypothesis was tested in NIH/3T3 fibroblasts using three complementary approaches to interfere with the function of normal and transforming versions of Raf-1. These strategies included expression of c-*raf*-1 antisense RNA or kinase inactive c-*raf*-1 mutants, as well as the analysis of a cellular mutant phenotype that can suppress v-*raf* transformation.

The first two approaches were used to determine whether Raf-1 is an essential component in receptor signaling mediated by serum growth factors. Serum was chosen for its pleiotropic action supplying both factors necessary for viability and long-term proliferation of fibroblasts. The major mitogen in serum is PDGF, which was previously shown to regulate Raf-1 kinase activity [4, 11]. As many growth factors also stimulate protein kinase C (PKC) [reviewed in 15], another serine/threonine kinase, activation of PKC by the tumor promoter TPA was examined as well. Different portions of mouse and human c-*raf*-1 cDNAs were expressed in both sense and antisense orientation using the retroviral expression vector pMNC (from Dr. B. Seed). This vector contains Moloney murine/leukemia virus (MoMuLV) long terminal repeats (LTRs), a neomycin resistance gene, and a human cytomegaly virus promoter that drives transcription of inserted sequences. As an initial screening assay these constructs were transfected into NIH/3T3 cells and the number of neomycin-resistant (neo^R) colonies was scored. Antisense constructs consistently yielded approximately twofold less

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colonies than the corresponding sense construct or the vector control plasmid indicating that expression of *raf* antisense RNA interferes with viability and/or proliferation of NIH/3T3 cells cultivated in the presence of 10% fetal calf serum. Based on our previous speculation that the Raf-1 regulatory domain interacts with activating ligands [14, 16], we reasoned that Raf protein inhibition might also be achieved through a competitive mechanism. Indeed, expression of a truncated Raf-1 protein, which encompasses most of the regulatory domain, reduced colony yield about fourfold. Expression of a mutant Raf-1 protein, cRaf301 (plasmid p301-1), that carriers an inactivating point mutation in the ATP binding site (lysine^{375} to tryptophan) was even more efficient, decreasing colony yield about sevenfold. This type of inhibition therefore most likely involves competition for activating ligands as well as Raf kinase substrates. Efforts to establish stable cell lines from the Raf inhibition experiments with NIH/3T3 cells repeatedly failed. The majority of cell clones died out and others lost expression of the transfected *raf* sequences after being kept in culture for six to ten passages.

Therefore, we turned to *raf* transformed cell lines where two parameters, morphological reversion and inhibition of proliferation, were studied. Transfection with the mutant *craf*301 expression plasmid efficiently reduced neo^R colony yield and moreover induced complete or partial reversion of the transformed phenotype in 25% or 35% of neo^R colonies, respectively. In both respects, expression of *raf* antisense RNA was less potent. It, however, yielded a range of cell clones with different morphologies that were stable enough to permit biochemical analysis. *raf*-transformed cells synthesize DNA even when serum-starved. The ability of morphological revertants to replicate DNA constitutively or in response to serum growth factors or TPA was reduced or eliminated in direct proportion to reduction in Raf protein levels (Fig. 1). In GMS-8/2, a cell clone that expresses low levels of v-Raf and Raf-1 proteins, constitutive DNA synthesis was decreased in starved cells, but still could be stimulated by TPA or serum. Further reduction of Raf protein levels in clone GMS-8/3 resulted in a substantial decline in serum and an almost complete loss of constitutive and TPA-inducible DNA replication. We conclude that, at least in NIH/3T3 cells, Raf-1 is an essential component of signal transduction pathways used by serum growth factors and PKC.

Given the strong evidence that Ras proteins are crucial regulators of signals arising at the cell membrane, this study was extended to *ras*-transformed cells. v-Ki-*ras*-transformed NIH/3T3 cells were transfected with the p301 plasmid series (Table 1). Judged by the neo^R colony yield, proliferation was impaired to a similar degree as seen in NIH/3T3 cells. Morphological reversion of *ras*-transformed cells, however, was about twofold less efficient than in *raf*-transformed cells. This difference could be due to secondary events that occured after transformation or else indicate that *ras*-induced transformation is only partially dependent on Raf-1. To test the effect of *raf* inhibition on the initiation of *ras* transformation a constant amount of v-Ha-*ras* (pSV2neo/*ras* [17], from Dr. T. Shih) plasmid was cotransfected with an equal or 4:1 molar excess of the p301 vectors (Table 2). Although the neomycin resistance marker of pMNC-based plasmids accounts for a background of flat neo^R colonies that presumably do not express pSV2neo/*ras*, it is clearly evident that transfection with p301 vectors causes an increase in morphologically reverted colonies at the expense of transformed colonies. The inhibition is dose-dependent and higher with the *craf*301 sense construct (p301-1). Transfected in a fourfold excess p301-1 almost completely interferes with the establishment of *ras* transformation indicating that Raf-1 function is necessary for *ras*-mediated proliferation as well as initiation of transformation.

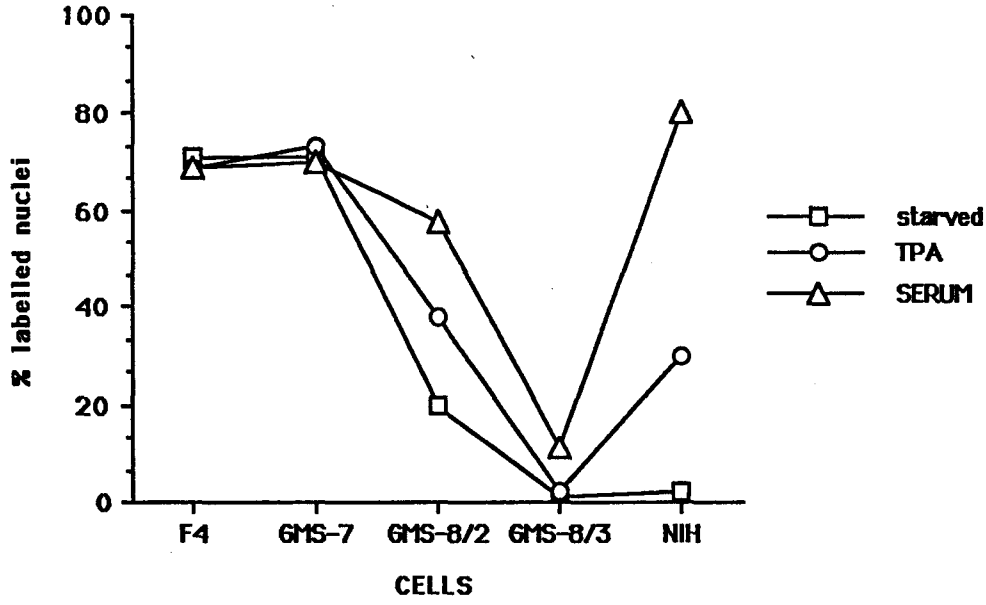

Fig. 1. Mitogen responsiveness of Raf-depleted cells. A 3611-MSV (v-*raf*)-transformed NIH/3T3 cell line, F4, was transfected with neo^R pMNC plasmids expressing *raf* antisense RNA targeted at the kinase domain (GMS-8) or a corresponding sense RNA (GMS-7). The analysis of neomycin-selected clones demonstrated that morphological reversion correlated strictly with down-regulation of v-raf and endogenous Raf-1 protein levels by antisense RNA, whereas expression of sense RNA showed neither effect. GMS-8/2 and GMS-8/3 are flat antisense induced revertant clones which, as judged by western blotting, express very low or undetectable levels of Raf proteins, respectively. GMS-7 represents a pool of ten clones transfected with the corresponding sense control plasmid. Selected clones were tested for mitogen responsiveness. DNA synthesis induced by serum or TPA in serum-starved cells is depicted as the number of nuclei incorporating [^{3}H] thymidine

The requirement of Raf function for transformation was more extensively studied in CHP25 cells. CHP25 is a flat revertant cell line that was generated from v-*raf*-transformed NIH/3T3 fibroblasts by selection with 4-*cis*-hydroxyproline, an amino acid analog which is significantly more toxic for fast growing, transformed cells than for normal NIH/3T3 cells [18, 19]. CHP25 cells express a functional v-*raf* oncogene, but are nontumorigenic, and do not form colonies in soft agar indicating that the revertant cell phenotype is due to acti-

Table 1. *raf* inhibition blocks *ras* mediated proliferation

Plasmids	Yield of neo^R colonies	Morphology of neo^R colonies		
		Flat (%)	Intermediate (%)	Transformed (%)
pMNC	197	0	0	100
pMNC301-2	120	2	15	83
pMNC301-1	58	15	15	70

V-Ki-*ras* transformed cells were transfected with the indicated plasmid DNAs, and neo^R colonies were microscopically examined for morphological reversion. pMNC301 are pMNC vectors which express the point-mutated c*raf*301 cDNA in either sense (p301-1) or antisense (p301-2) orientation. Percentages are calculated based on examination of a total of ≥ 200 colonies per transfection.

Table 2. *raf* inhibition blocks *ras* transformation

Plasmids	Inhibition	Morphology of neo^R colonies		
	(%)	Flat (%)	Intermediate (%)	Transformed (%)
Ratio 1:1				
ras + pMNC	0	27	17	56
ras + p301-2	53	28	46	26
ras + p301-1	61	46	32	22
Ratio 1:4				
ras + pMNC	0	33	23	44
ras + p301-2	61	48	35	17
ras + p301-1	84	67	25	7

NIH/3T3 cells were co-transfected with v-Ha-*ras* (pSV2 noe/ras) and p301.

vation of a cellular suppressor function. The variant phenotype presumably resulted from a single mutation, since retransformants could readily be isolated from cultures growing at high cell densities. CHP25 cells are resistant to transformation by *sis*, *ras*, and tyrosine kinase as well as serine/threonine kinase family oncogenes suggesting that Raf functions downstream of most peripheral signal transducers. In contrast to v-*raf*-transformed cells, in which the endogenous Raf-1 protein kinase is constitutively activated, v-Raf in CHP25 cells did not activate endogenous Raf-1 kinase. Since mitogen regulation of Raf-1 kinase in CHP25 cells remained intact CHP25 cells seem to be blocked at the level of Raf-1 substrate phosphorylation. Consistent with this interpretation, CHP25 cells show specific alterations of early gene induction. The serum induction of c-*fos* and *jun*D as well as the serum and TPA induction of *jun*B and *egr*-1 are almost completely abolished. These data demonstrate that Raf-1 signaling is essential for transformation of NIH/3T3 cells by peripheral oncogenes and for regulation of a subset of early-response genes by TPA and serum growth factors. The availability of a permanent cell line, CHP25, that is blocked at the level of Raf function should greatly facilitate the dissection of this pleiotropic *raf*-dependent gene response pattern as well as help to identify raf substrates which mediate *raf*-regulated mitogenesis.

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