The carboxy-terminal catalytic domain of the GTPase-activating protein inhibits nuclear signal transduction and morphological transformation mediated by the CSF-1 receptor

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To determine whether ras p21 products are necessary for signal transduction mediated by the colony stimulating factor-1 receptor (CSF-1R, the c-fms proto-oncogene product), we determined whether CSF-1R and ras activate a common nuclear target and whether the interruption of ras action affects CSF-1R signal transduction. Expression of the NVL3 retrotransposon was activated to the same extent in NIH-3T3 cells by both ras and v-fms oncogenes, and the ras-responsive element located in the long terminal repeat of NVL3 was demonstrated to be a common target for oncogene action. Human recombinant CSF-1 stimulated expression of the NVL3 element 30-fold in NIH-3T3 cells that contained human CSF-1R. Expression of the carboxy-terminal 374 amino acid residues of the human ras GTPase-activating protein (GAP) in cells containing CSF-1R was able to inhibit CSF-1 induction of NVL3 expression by 90%. Expression of the catalytic domain of GAP was also able to suppress transformation by either v-fms or ligand-activated CSF-1R. Expression of the c-jun proto-oncogene was activated by CSF-1R but was insensitive to the action of the catalytic domain of GAP. These results provide genetic evidence that in NIH-3T3 cells, ras p21 is involved in signal transduction mediated by CSF-1R.

[Key Words: Colony stimulating factor-1 receptor; ras oncogene; GTPase-activating protein; signal transduction; transcriptional regulation]

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The p21 ras products can be viewed as molecular switches in signal transduction pathways important for cell proliferation and differentiation [Babacik 1987]. The ras–guanosine triphosphate (GTP) complex is active in signal transduction, whereas the ras–guanosine diphosphate (GDP) complex is inactive, and mutations that abrogate ras GTPase activity activate its oncogenic potential. Genetic and biochemical evidence in budding yeast demonstrates that ras proteins regulate cAMP levels [Toda et al. 1985]; however, in mammalian cells, both the upstream cell-surface receptors that couple to ras and its downstream effectors remain less well defined [for a recent review, see Candley et al. 1991].

The GTPase-activating protein (GAP) plays an important role in regulating ras action. This protein stimulates the GTPase activity of wild-type ras proteins in vitro and forms a complex with activated ras. Activated ras GTPase activity, however, is not stimulated by GAP [Trahay and McCormick 1987]. Overexpression of GAP inhibits transformation of cells by wild-type Harvey [Ha]-c-ras but not transformation mediated by mutant ras alleles (Zhang et al. 1990), suggesting that ras GAP functions as a negative regulator of signal transduction. GAP overexpression can also inhibit cellular transformation by the v-src oncogene, indicating that endogenous ras is required for v-src transformation and supporting a role for GAP as a negative regulator of ras action [DeClue et al. 1991; Nori et al. 1991].

To define signal transduction pathways that link ras action to nuclear targets, we have focused on the selective alteration of transcription of cellular genes by activated ras [Owen and Ostrowski 1987, 1990; Owen et al. 1990]. In this study we have asked whether the colony stimulating factor 1 receptor (CSF-1R) activates the same nuclear targets as mutated ras alleles and whether the interruption of ras action affects CSF-1R signal transduction.

The CSF-1 receptor is encoded by the c-fms proto-oncogene [Sherr et al. 1985] and is one of a family of growth...
factor receptors with intrinsic protein tyrosine kinase (PTK) activity (Sherr 1990). When the human receptor is introduced into NIH-3T3 cells, its activation by CSF-1 leads to morphological cell transformation (Roussel et al. 1987, Roussel and Sherr 1989). In addition, point mutations in the receptor gene that constitutively activate its PTK activity lead to factor-independent cell transformation (Roussel et al. 1988; Woolford et al. 1988). Microinjection of anti-ras monoclonal antibodies into v-fms-transformed cells can cause reversion of the transformed phenotype (Smith et al. 1986), suggesting that ras may function in a signal transduction pathway downstream of fms. In accord with these results, CSF-1 treatment of NIH-3T3 cells that express human CSF-1R results in a threefold increase in the amount of GTP complexed to ras p21 (Gibbs et al. 1990). GAP and GAP-associated proteins can also be phosphorylated on tyrosine and bind to CSF-1R in response to CSF-1 stimulation of receptor-containing rat cells (Reedijk et al. 1990).

We provide further molecular and genetic evidence that support a role for ras in a CSF-1R signaling pathway. Expression of the mouse NVL3 retrotransposon, a target of ras action (Owen et al. 1990; Owen and Ostrowski 1990), is up-regulated in cells containing activated CSF-1 receptors. A common NVL3 enhancer element is the target for the action of both v-fms and ras. When the carboxy-terminal catalytic portion of human GAP (Marshall et al. 1989) is introduced into cells expressing CSF-1R or v-fms, activation of the NVL3 gene is inhibited and morphological transformation is suppressed. These results indicate that CSF-1R signal transduction is dependent, at least in part, on the action of ras products.

Results

The NVL3 ras-responsive element is activated in v-fms-transformed cells

The mouse VL30 retrotransposon NVL3 is activated in ras-transformed cells (Owen and Ostrowski 1987). We used an S1-mapping assay with an NVL3-specific probe to test whether NVL3 was also activated in cells transformed by the v-fms oncogene (Fig. 1). By these criteria, NVL3 RNA expression is activated to the same extent in v-fms- and ras-transformed cells (Fig. 1; v-fms and DT, respectively), but its expression is very low in normal NIH-3T3 cells. One potential problem with this experiment is the specificity of the S1 assay for the NVL3 gene. The VL30 element family contains ~100 members (for review, see Keshet et al. 1990). Can we be sure that our assay detects only NVL3 expression? We have recently determined the nucleotide sequence of different VL30 U3-R long terminal repeat (LTR) elements obtained by polymerase chain reaction (PCR) amplification (M.S. Colman and M.C. Ostrowski, unpubl.). In addition, the sequences of other independently derived VL30 elements have been reported [e.g., Itin and Keshet 1986; Schiff et al. 1991]. Their analysis reveals that individual VL30 elements contain many single-base differences in the R region; even closely related elements that are reg-
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Table 1. Activation of the NVL3 ras-responsive element in v-fms-transformed cells

|           | 3T3<sup>b</sup> | v-fms 3T3<sup>b</sup> |
|-----------|-----------------|------------------------|
| pRDO51<sup>a</sup> | 1.15 ± 0.66     | 1.77 ± 0.36            |
| pRDO53    | 3.77 ± 2.39     | 33.93 ± 7.14           |
| Ratio 53/51 | 3.3             | 19.2                   |

<sup>a</sup>For each experiment, 9 µg of reporter and 1 µg of secreted alkaline phosphatase expression vector (internal control, see Materials and methods) were cotransfected. Results represent the average of three independent experiments, each performed in duplicate.

<sup>b</sup>Values are relative luciferase activity, presented as luminescence/µg of cellular protein/mU of SEAP/ml of culture media.

target of CSF-1R, we examined expression of NVL3 in NIH-3T3 cells that contained the wild-type receptor. CSF-1 activates the PTK activity of human CSF-1R expressed in NIH-3T3 cells, and growth factor treatment results in cell proliferation (Roussel et al. 1987; Roussel and Sherr 1989). We used the S1-mapping assay to determine whether ligand activation of the PTK activity of CSF-1R could lead to an increase in NVL3 expression. Figure 2A shows that the addition of human recombinant CSF-1 to cells expressing CSF-1R resulted in a 30-fold increase in NVL3 RNA levels. Augmented NVL3 expression was seen within 30 min after growth factor addition, and maximal stimulation was seen after 16 hr of treatment. The final level of NVL3 expression was the same as that seen in cells transformed by ras (Fig. 2A, cf. lanes 24 and DT). Expression of NVL3 was maximally elevated even after 72 hr of growth factor stimulation [data not shown]. For these and subsequent experiments, the expression of the pSV2-neo gene within these cells was used as an internal control for the S1-mapping assay (Fig. 2A, bottom). CSF-1 had no effect on NVL3 expression in NIH-3T3 cells that lacked human CSF-1R [data not shown].

To ascertain whether induction of NVL3 expression by CSF-1R depended on post-translation modification of cellular proteins or whether de novo protein synthesis was required, the effect of the protein synthesis inhibitor cycloheximide was evaluated (Fig. 2B). Concurrent treatment of receptor-positive cells with cycloheximide and CSF-1 inhibited stimulation of NVL3 expression when assayed after either 1 or 8 hr of treatment. Cyclohexim-
To determine whether the induction of NVL3 expression was a general property of PTK receptors, we tested the ability of another PTK receptor to stimulate NVL3 transcription. The genes encoding CSF-1R and the β-receptor for platelet-derived growth factor [PDGF] are related [Yarden et al. 1986] and appear to be derived from a common ancestral gene [Roberts et al. 1988]. Therefore, we chose to study the effects of PDGF on NVL3 expression in NIH-3T3 cells that contain both exogenous CSF-1R and endogenous PDGF-R [Fig. 2C]. The effect of PDGF (BB isoform) stimulation was similar to that of CSF-1 for 8 hr, but PDGF was not able to induce the further increase in NVL3 expression observed between 8 and 24 hr with CSF-1. PDGF also stimulated NVL3 expression in an identical manner in NIH-3T3 cells that lacked CSF-1R [data not shown].

We also studied the expression of two immediate early genes after stimulation of cells with CSF-1 and PDGF [Greenberg and Ziff 1984; Ryder and Nathans 1988]. Figure 3A shows that c-fos was rapidly and transiently induced following treatment of cells containing human CSF-1R with either growth factor. Likewise, PDGF and CSF-1 stimulated c-jun in these cells [Fig. 3B], with expression observed within 30 min after growth factor treatment.

**The catalytic domain of GAP can inhibit transformation and nuclear signaling by CSF-1R**

To test whether endogenous ras proteins are involved in CSF-1R signal transduction events, we overexpressed the carboxy-terminal catalytic portion of GAP [Marshall et al. 1989] in cells containing CSF-1R. For this purpose, we cloned amino acid residues 673–1047 of the human GAP gene by PCR amplification of cDNA prepared from the bladder carcinoma cell line EJ and placed the segment downstream of the human β-actin promoter (see Fig. 4A). The PCR-amplified segment was sequenced in its entirety and was found to be identical to the published sequence (Trahey et al. 1988). The methionine codon at residue 673 serves as an initiation codon for the truncated product. The GAP expression vector was cotransfected with Val12-Ha-c-ras or with v-fms into NIH-3T3 cells, and the number of transformed foci were scored. In control experiments, both oncogenes were cotransfected with the expression vector lacking the GAP sequences. The results of two experiments are summarized in Figure 4B. The GAP construct had no significant effect on mutant ras transformation, in agreement with published observations [Zhang et al. 1990]. However, GAP expression blocked v-fms transformation by 50%. The ratio of GAP/oncogene was 10:1 in experiment 1 and 20:1 in experiment 2.

The GAP expression vector was introduced into established v-fms-transformed cells by cotransfection with a hygromycin-resistance gene [these cells already contained the neomycin-resistance gene [Roussel et al. 1984]]. After selection, 63 of 180 of the drug-resistant colonies (35%) were scored as having a flat, nontransformed morphology [data not shown]. In control experiments, expression vector lacking GAP and the hygromycin marker were cotransfected. In this case, no revertants were scored among ~100 colonies analyzed [data not shown]. These results suggest that GAP can suppress v-fms transformation. Both transformed and revertant drug-resistant colonies were picked and expanded from the GAP cotransfection experiments. Molecular analysis of these clones revealed three classes of transformants, represented by the three clones analyzed in Figure 5; (1) flat clones that overexpress the predicted 1.9-kb GAP RNA, represented by clone 6; (2) clones having a transformed morphology that express lower levels of 1.9-kb GAP RNA, as observed in clone 10. [When the relative amount of GAP RNA in these two clones was quantitated by densitometric scanning, clone 6 was found to express approximately three times the GAP RNA expressed by clone 10 (Fig. 5C)]. (3) transformed lines that express no GAP RNA, such as clone 2. All three classes of clones expressed equivalent amounts of v-fms mRNA, whereas the levels of NVL3 RNA expressed varied [Fig. 5A]. The S1 nuclease assay was used to quantitate more precisely the levels of NVL3 RNA in these v-fms/GAP clones [Fig. 5B]. Such experiments indicated that clone 6 expressed 27% of the level of NVL3 found in fms-transformed cells, whereas the level of NVL3 RNA in clone 10 and clone 2 were 50% and 100% of wild type, respectively [Fig. 5B,C]. In control experiments, we found that expression of the catalytic domain of GAP had no effect on NVL3 transcription in v-ras-transformed DT cells.

Figure 3. Induction of immediate early genes by CSF-1 and PDGF. Cells were either treated with 2000 U/ml of CSF-1 [left], or 2 nm PDGF [right] for 0, 0.5, 1, 2, or 8 hr. Total cellular RNAs were isolated and analyzed by Northern blotting [5 μg/lane] with human c-fos [A] or human c-jun [B] probes.
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dicated that in the presence of optimal levels of CSF-1, GAP decreased the number of soft agar colonies by 50% relative to cells containing CSF-1R alone. Colonies formed by cells containing both CSF-1R and GAP were much smaller than the colonies formed by CSF-1R alone. The effect on colony formation was even more marked when the cells were grown in the presence of lower concentrations of CSF-1 (Table 2). Control experiments demonstrated that an expression vector lacking GAP had no effect on the growth of CSF-1R-expressing colonies or on NVL3 RNA induction by CSF-1 (data not shown). Figure 6A shows a Northern blot containing RNA isolated from the CSF-1R/GAP cells and confirms that the 1.9-kb truncated GAP mRNA was produced.

Discussion

The NVL3 gene, a nuclear target of ras action (Owen and Ostrowski 1987; Owen et al. 1990), is also activated by v-fms and human CSF-1R in NIH-3T3 cells. Kinetic analysis of CSF-1R stimulation of NVL3 expression demonstrated that the maximal effect occurred 16–24 hr after the addition of hormone (Fig. 2) and remained elevated following 48–72 hr of growth factor treatment (data not shown). Inhibition of protein synthesis blocked CSF-1R stimulation of NVL3 expression but had no effect on basal NVL3 expression in the absence of CSF-1. Thus, stimulation of NVL3 expression by CSF-1R is distinct from the immediate early responses activated by CSF-1R and by other tyrosine kinase receptors (Greenberg and Ziff 1984; Ryder and Nathans 1988) and, instead, reflects a persistent effect on gene expression that parallels more closely morphological transformation of NIH-3T3 cells.

To determine whether ras gene products are required for CSF-1R-mediated nuclear signal transduction, the catalytic domain of the GAP protein (Marshall et al. 1989) was introduced into cells containing human CSF-1R. A similar strategy has been used recently to demonstrate that ras is required for v-src-mediated transformation of NIH-3T3 cells (DeClue et al. 1991; Nori et al. 1991). The carboxy-terminal domain of GAP quantitatively inhibited CSF-1R-induced NVL3 gene expression by 90%, as well as CSF-1R-dependent and v-fms-induced transformation of NIH-3T3 cells. These results, taken together with biochemical data showing that the amount of ras–GTP complex rapidly increases following
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Figure 5. Analysis of v-fms GAP clones. (A) Total cellular RNAs were isolated from v-fms GAP clones 2, 6, and 10. Northern blot analysis was performed (5 μg/lane) with NVL3, GAP, and v-fms probes, as indicated. (B) S1 nuclease mapping was performed with 10 μg of each RNA sample and the NVL3 probe. Clone designations are above lanes; the arrow depicts the protected fragment. (C) Comparison of relative NVL3 and GAP RNA levels in v-fins GAP clones. NVL3 RNA levels in v-fins or v-fins~GAP clones were quantitated from S1 assays (see Materials and methods). The amount of v-fins RNA is set as 100. GAP RNA levels were determined by densitometric scanning of autoradiographs of the Northern blots (see Materials and methods). The amount of GAP RNA in clone 6 is set as 100.

Activation of CSF1-R (Gibbs et al. 1990), lead to the conclusion that rasp21 is a component of a signal transduction pathway in NIH-3T3 cells relevant for CSF-1 action.

PTK receptors appear capable of activating multiple signal transduction pathways (for review, see Cantley et al. 1991). In contrast to the results obtained for the NVL3 gene, expression of the catalytic domain of GAP does not affect CSF-1 stimulation of c-jun mRNA expression. A simple interpretation of this result is that GAP can selectively interfere with the ability of the receptor to activate individual effector pathways. Similarly, a point mutation in CSF-1R impairs its ability to stimulate a mitogenic response while having no effect on its association with phosphatidylinositol 3-kinase or its stimulation of c-fos and junB gene expression (Roussel et al. 1990). Although our results suggest that CSF-1 stimulation of c-jun expression does not rely on ras, more experimental evidence is required to demonstrate conclusively that activated ras does not stimulate c-jun mRNA expression. A recent report has presented data suggesting that oncogenic ras can increase c-jun activity by a post-translational mechanism (Binetruy et al. 1991). Taken with the simple explanation for our results, this indicates that PTK receptors may use multiple signaling pathways to activate a single gene product and that distinct levels of regulation can be the target of these pathways. Thus, although effects of a distinct pathway may be resolved by approaches similar to those reported here, the biological activity of the PTK receptor may depend on the interaction of several signaling pathways.

CSF-1 stimulated NVL3 expression fivefold more efficiently than PDGF in cells that contain the CSF-1R. There are more PDGF- than CSF-1-binding sites on these cells (Bowden-Pope and Ross 1982; Roussel and Sherr 1989), so that the difference in response is probably not the result of the relative levels of receptor expression. Although the receptors for these growth factors are very closely related (Yarden et al. 1986; Roberts et al. 1988), and activation of either receptor leads to a rapid threefold increase in ras-GTP complex in NIH-3T3 cells (Gibbs et al. 1990), they have distinct biological properties. For instance, under our growth conditions, exogenously added PDGF alone cannot stimulate DNA synthesis or induce morphological transformation of NIH-3T3 cells (Roussel et al. 1987; Roussel and Sherr 1989). Our data imply that CSF-1R activates ras-dependent signaling pathways more efficiently, or in a different manner, than the PDGF receptor, so that this property may account for the different biological activities observed.

GAP is a negative regulator of ras action, but it is possible that GAP is also an effector of ras signal trans-

Table 2. Effect of expression of the catalytic domain of GAP on the cloning in semisolid media of NIH-3T3 cells expressing the CSF-1 receptor

| Cell lines | −CSF-1 | +CSF-1 |
|------------|--------|--------|
| CSF-1R 3T3 | 0.3*   | 30.2   |
| CSF-1R + GAP | 0.0    | 18.05 |

*Numbers represent the efficiency of cloning in semisolid medium, i.e., the number of colonies counted divided by the total number of cells plated. The average of two independent experiments is presented. Experiments were scored 2 weeks after plating.

The size of the CSF-1R/GAP-expressing colonies was reduced compared to CSF-1R-expressing colonies.
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Figure 6. Analysis of GAP, NVL3, and c-jun expression in CSF-1R GAP cells. (A) CSF-1 treatment of CSF-1R and CSF-1R-GAP cells was performed as described in the legend to Fig. 2 for 0, 2, 8, or 24 hr. Total cellular RNAs were isolated and analyzed by Northern blotting (10 μg/lane) with a GAP probe. The arrow depicts the position of the 1.9-bp GAP RNA species. (B) S1 nuclease mapping was performed on 10 μg of each RNA sample described in A, with NVL3 (top) or SV-neo (bottom) probes. Control lanes include DT RNA or tRNA. Arrows indicate protected fragments for NVL3 and SV-neo RNAs, respectively. (C) Northern blotting analysis was performed with the c-jun probe on a duplicate blot of that utilized in A. The blot was stripped and rehybridized with K-rev-1 probe (bottom) to control for differences in sample loading.

We provide molecular and genetic evidence that a specific tyrosine kinase receptor, the CSF-1R, is coupled to a ras-dependent signaling pathway. The biological importance of this ras pathway in cell types other than NIH-3T3 remains to be determined, but the general approach and reagents employed here should be useful for ascertaining whether the ras pathway is operative in other types of cells. Further molecular characterization of the ras pathway important for CSF-1R action should clarify how ras products participate in cellular transformation, as well as the role of these proteins in normal cell growth and differentiation.

Materials and methods

Mammalian cell lines

Cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 9.5 mM L-glutamine, and 100 μg/ml of mezlocillin. Cells lines included NIH-3T3, ras-transformed DT cells (Owen et al. 1990), NIH-3T3 expressing human CSF-1R (Roussel and Sherr 1989), and NIH-3T3 transformed with v-fms (Roussel et al. 1984). To generate cells that coexpress the CSF-1 receptor and the carboxy-terminal portion of GAP, NIH-3T3 cells were cotransfected with a human c-fms expression vector and the p[APR-GAP expression vector (see below). The cells were selected in 800 μg/ml of G418 and then subjected to FACS analysis with monoclonal antibodies directed to extracellular epitopes of human CSF-1R (Roussel and Sherr 1989). Soft agar colony assays were performed as described previously (Roussel et al. 1987). For selection of v-fms revertants, v-fms-transformed cells were cotransfected with p[APR-GAP and a Moloney leukemia virusLTR-hygroycin-resistance gene (Blochlinger and Diggelmann 1984). Cells were selected for 2 weeks in 160 μg/ml of hygromycin (Calbiochem), and flat revertants were cloned in microcylinders and expanded. Recombinant human CSF-1 was a gift from Steven C. Clark (Genetics Institute, Cambridge, MA). CSF-1 treatment of cells was performed as described previously (Roussel and Sherr 1989). For experiments reported here, 2000...
U/ml of CSF-1 was added to the cell media (1 U = 0.44 fmole). Human recombinant PDGF [BB isoform] was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY).

**Plasmids**

Plasmid pBAPR–GAP contains amino acids 673–1047 of the human GAP gene under the control of the ~-actin promoter. Plasmid pBAPR–GAP contains amino acids 673–1047 of the human GAP gene under the control of the ~-actin promoter.

**Transfections**

Procedures for transfections of these plasmids into mammalian cells have been described previously [Owen et al. 1990]. Focus assays were scored after 14 days for ras and after 21 days for v-fms. For transient assays, the secreted alkaline phosphatase gene was included as internal control for transfection efficiency [Owen et al. 1990]. Luciferase activity in transient assays is presented as luminescence per microgram of protein extract divided by secreted alkaline phosphatase activity per milliliter of culture media.

**Isolation and analysis of RNA**

RNA was isolated as described previously [Iverson et al. 1987]. The procedure for high-stringency S1 mapping has been described previously [Owen and Ostrowski 1987]. For detection of pSV2–neo RNA present in the cells, a 419-bp probe, generated by digestion of pSV2–neo (Southern and Berg 1983) with Neol and BgIII, was used. This probe was end-labeled at the BgIII site and protected a 377-base neo RNA fragment (e.g., see Fig. 2B). For quantitation of S1 results, bands were excised from gels, and the amount of radioactivity was measured by Cerenkov counting. The Northern blotting procedure has also been described previously [Owen and Ostrowski 1987], except 5–10 µg of total cellular RNA was used per lane in the denaturing agarose gel. The probes were labeled by the random priming technique with a kit supplied by Boehringer Mannheim and following protocols supplied by the manufacturer. RNA levels detected by autoradiography were quantitated with a laser scanning densitometer obtained from Biomed Instruments, Inc. (Ful lerton, CA).

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