8. Sizable crystals of $C_{60}Br_{24} \cdot Br_2$ were grown directly from a reaction mixture consisting of C_{60} (35 mg) and Br_2 (5 ml) in a Teflon FEP tube tightly capped under nitrogen. Initially, the mixture was cooled from 70° to 65°C over 5 hours, and then to 55°C over 20 hours. The temperature was increased rapidly to 65°C and allowed to cool over 20 hours to 55°C. The latter routine was repeated, and finally the system was cooled from 65° to 25°C over 70 hours. An orange plate with dimensions of 0.16, 0.21, and 0.08 mm was mounted on a Syntex P3 diffractometer (graphite-monochromatized MoKα radiation, λ = 0.71069 Å) and cooled to −130°C. The rhombohedral unit cell parameters (in hexagonal form) determined from the Bragg angles of 50 computer-centered reflections are a = 12.874(2) and c = 32.659(6) Å. Six octants of intensity data were collected ($2\theta < 60°$), corrected for absorption (numerical integration: transmission factors ranged from 0.10 to 0.26), and finally averaged. The space group was assigned as $R\bar{3}$ (no. 148); the structure later confirmed the assignment. The structure was solved by direct methods and was refined by full-matrix least-squares techniques. The initial refinement of the $C_{60}Br_{24}$ molecule converged very quickly; a difference map at this point clearly indicated the presence of additional atoms that were assigned as Br_2 molecules of solvation (see text). Various disorder models were used to fit this extra density but the most successful included 14 sites distributed about the $\bar{3}$ symmetry axis located at the origin of the cell: two sites [Br(5)] were on the threefold axis itself, each of which were assigned an occupancy of 0.5; 12 other sites [Br6′ and Br6″] in general positions were assigned occupancies of 0.083. Attempts were made to refine the occupancies but the high correlation between the occupancies and the thermal parameters of the Br_2 atoms caused the least-squares refinement either to diverge or to converge to totally unacceptable values. The final refinement of 88 parameters (Br1 to Br5 with anisotropic thermal parameters; Br6′, Br6″, and C with isotropic thermal parameters; Table 1) in which 1229 unique reflections with $I > 2.5\sigma(I)$ were used converged at R = 0.039 and Rw = 0.038. The final difference map was featureless. The possibility of synthesizing more than one type of brominated species led to an examination of the reaction product by x-ray powder diffraction. The observed diffraction pattern was then compared with the "expected" pattern calculated from the single-crystal structure of $C_{60}Br_{24} \cdot Br_2$. The two patterns were found to be similar; the single crystal was clearly representative of the bulk product for these reaction conditions. Powder patterns of other samples have been observed to vary with synthesis conditions.
9. Carbons bonded to metal complexes and to the osmate group (*2, 4*) are also "pulled away" from the surface of the C_{60} cage.
10. Few molecules have T_h symmetry and the complete character table for this point group is sometimes omitted from textbooks. It is included in F. A. Cotton, *Chemical Applications of Group Theory* (Wiley, New York, ed. 2, 1971). (There is a misprint in the character of the inversion operation for the T_g and T_u representations.)
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16. The extent to which the formation of $C_{60}Br_{24}$ is disfavored depends on the number of isomers and symmetry of $C_{60}Br_{22}$.
17. We thank J. Sasson for his perspective on the chemical reactivity of Br_2; W. J. Marshall for skilled x-ray technical assistance; E. Pascher (Remagen) for elemental analyses; and D. A. Dixon, T. Fukunaga, J. Y. Becker, P. J. Krusic, and P. J. Fagan for helpful discussions. Contribution no. 6144.

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Implication of GAP in Ras-Dependent Transactivation of a Polyoma Enhancer Sequence

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Controversy exists as to whether the interaction of a guanosine triphosphatase activating protein (GAP) with Ras proteins functions both to initiate and to terminate Ras-dependent signaling events or only to terminate them. GAP-C, a carboxyl-terminal fragment of GAP that is sufficient to stimulate GTPase activity, inhibited the stimulation of transcription produced by some oncoproteins (v-Src, polyoma middle T, wild-type Ras, and oncogenic Ras) but not that produced by v-Mos. Wild-type GAP did not affect transcription induced by oncogenic Ras but reversed the inhibitory effect of GAP-C on transcription induced by oncogenic Ras. These results indicate that GAP is a negative regulator of wild-type Ras and elicits a downstream signal by interacting with Ras-GTP (guanosine triphosphate).

The function of GAP was investigated by studying its effect on the transcription from the polyoma virus–thymidine kinase (Py-TK) promoter, which is strongly activated by expression of Ras in Chinese hamster ovary (CHO) cells. Cells were cotransfected with a plasmid containing the chloramphenicol acetyl transferase (CAT) gene under the control of the Py-TK promoter (Py-TK-CAT), and expression vectors containing an oncogene and either the human GAP gene or the nucleotide sequence encoding its catalytic domain (amino acids 702 to 1044) under the control of the SV40 early promoter.

A common motif similar to the binding site for a protein (PEA1) that binds to the polyoma virus enhancer (GAGTTAGTCAC) is the target of action for oncoproteins such as v-Src, polyoma middle T, Ras, and v-Mos. In CHO cells all of these oncoproteins activated such an element containing four head-to-tail copies of the PEA1 motif from the polyoma virus enhancer (Fig. 1). Oncogenic Ras (Ha-Ras-Val^{12}) increased the rate of transcription from this element up to 15-fold. There is a good correlation between the capacities of various oncogenes to activate transcription from PEA1 and to transform cells (*1, 2*); furthermore, the loss of transactivation of the PEA1 motif correlates with phenotypic reversion (*3*). Oncogenic Ras also induces binding of nuclear proteins to a PEA1 motif

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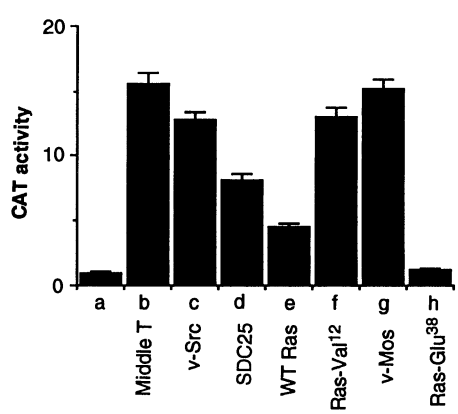

Fig. 1. Oncoprotein activation of an element from the polyoma virus enhancer. The cDNA sequences corresponding to the different transactivating proteins were inserted in an expression vector (SV2) downstream of the SV40 early promoter and enhancer (*1*). These plasmids were transfected into CHO cells with another vector containing CAT gene under the control of a Py-TK promoter (Py-TK-CAT). This synthetic promoter contains four head-to-tail copies of the PEA1 binding site of the polyoma enhancer upstream of the TK promoter. Lipospermine (Transfectam, IBF-SEPRACOR) was used as a transfecting agent. CHO cells (2×10^6) were transfected with Py-TK-CAT (0.5 μg) and 0.1 μg of the appropriate expression vector. Total DNA was adjusted to 5 μg with expression vector without insert. CAT activity was determined 48 hours after transfection and culture in Dulbecco's minimum essential medium (DMEM) supplemented with fetal calf serum (1%) as described (*32*). Data were recorded in arbitrary units; the basal signal due to activity of the reporter gene alone was assigned a value of 1. Columns a through h represent CAT activity in cells transfected with: (a) Py-TK-CAT alone, (b) sequences encoding polyoma middle T, (c) v-Src, (d) SDC25-C (*11*), (e) wild-type Ha-Ras, (f) Ha-Ras-Val^{12}, (g) v-Mos, or (h) Ha-Ras-Val^{12} Glu^{38}. The data are means of three independent experiments normalized to the CAT activity generated in the presence of the reporter gene alone.

(4) and mutations within the so-called effector domain on Ras ($Thr^{35} \rightarrow Ala$, $Asp^{38} \rightarrow Glu$) abolish proper signal transduction. Our data reinforce the idea that the integrity of this domain is critical for transactivation (4) (Fig. 1). Ras with a mutation at Glu^{38} is insensitive to stimulation by GAP and biologically inactive (6). Thus, GAP may participate in Ras-induced transactivation.

Expression of GAP inhibited the activation of the Ras-responsive element that resulted from expression of polyoma middle T, v-Src, the COOH-terminus of SDC25 (SDC25-C) (a *Saccharomyces cerevisiae* guanine nucleotide exchange factor for Ras proteins), and wild-type Ras (Fig. 2). GAP-C, a COOH-terminal fragment of GAP, containing amino acids 702 to 1044, inhibited transactivation elicited by the same oncoproteins (5) and that elicited by the oncogenic Ras (Fig. 2). Neither GAP nor GAP-C modified transactivation by v-Mos. This result is in accordance with reports showing that Mos acts independently of the Ras-GTP effector complex (8, 9).

GAP and GAP-C inhibited transcriptional activation of the PEA1 motif by polyoma middle T, SDC25-C, and v-Src to a greater extent than that by wild-type Ras. One of the cellular functions of middle T is to mobilize $pp60^{src}$, possibly through activation of tyrosine phosphatases (7). Activation of $pp60^{src}$ precedes activation of Ras in the sequence of biological events leading to transformation, as judged by microinjection studies (8). In NIH 3T3 cells, v-Src activates Ras, resulting in increased GTP binding. The ratio of GTP:GDP (guanosine diphosphate) bound to Ras is greater in cells expressing v-Src (10) or SDC25-C (11) than in cells expressing c-Ras (10, 11). Therefore it is not surprising that expression of GAP or GAP-C, which inactivate GTP-bound Ras, has a more pronounced effect on transcription induced by middle T, v-Src, or SDC25-C than on transcription induced by wild-type Ha-Ras or endogenous c-Ras (Fig. 2). Overexpression of either GAP or GAP-C inhibits transformation by wild-type Ras (12) or v-Src (13, 14) and decreases the amount of Ras bound to GTP in NIH 3T3 cells (10).

Expression of GAP does not affect the transcriptional effects of activated Ha-Ras-Val^{12} because the GTPase activity of oncogenic Ras is not stimulated by GAP. However, GAP-C had an antagonistic effect on the oncogenic Ras signaling pathway (Fig. 2). GAP-C inhibited the effect of Ha-Ras-Val^{12} to a greater extent than that of wild-type Ha-Ras (Fig. 2). This result is not expected if GAP-C acts catalytically to decrease wild-type Ras signal transduction and if it competitively blocks the effect of oncogenic Ras. The difference was no longer apparent when we increased the amount of wild-type Ha-Ras transfected (and thus the total amount of active Ras-GTP). Under these conditions, GAP-C inhibited the effect of wild-type Ras and v-Src equally (15). Our data are therefore most consistent with a model in which GAP-C acts catalytically to switch off the wild-type Ras-GTP complex and competes with endogenous Ras effectors to block the binding of oncogenic Ras-GTP. One such effector may be GAP itself. Mutational analysis has revealed that amino acids of Ras essential for GTPase stimulation by GAP are also essential for biological activity (16, 21). GAP also binds to all oncogenic Ras proteins, although it has no effect on their GTPase activity. Other evidence of the participation of GAP in the effector complex includes the demonstration that addition of GAP to Ras blocks muscarinic K^+ channels in atrial cells (17). GAP-C is not sufficient for channel blocking (17). Other reports suggest that GAP is an upstream regulator of Ras proteins (12, 18–20). Sequence analysis of GAP has not revealed any clues as to whether it has an effector function. Our data are also consistent with GAP-C competing with another effector molecule binding to the same site on Ras.

To test this hypothesis, CHO cells were cotransfected with GAP and GAP-C (Fig. 3). Expression of GAP reversed the negative transcriptional effects of GAP-C (Fig. 3A) in the presence of oncogenic Ras. Thus GAP and GAP-C appear to compete for binding to Ras and GAP participates in producing the downstream signals elicited by oncogenic Ras. Cotransfection with GAP does not reverse the negative transcriptional effect of GAP-C when wild-type Ras (Fig. 3B), polyoma middle T antigen, v-Src, or SDC25-C is overexpressed (15). As a control, neither GAP nor GAP-C modified v-Mos downstream signals (Fig. 3C). It is unlikely that GAP-C interacts with a guanine nucleotide exchange factor for Ras proteins because GAP and GAP-C both inhibit the activation of the Py enhancer that results from expression of SDC25-C.

Because GAP does not modify Ha-Ras-Val^{12} signaling, but GAP-C does, it appears

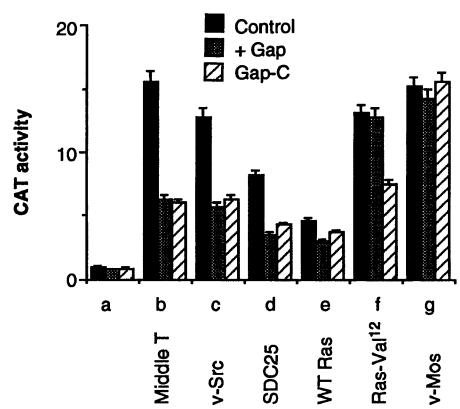

Fig. 2. Effect of GAP and GAP-C on transactivation of Py-Tk-CAT by various oncoproteins. Each column corresponds to the CAT activity obtained when CHO cells were transfected with the CAT construct (0.5 μg) the vector encoding transactivating protein (0.1 μg) and empty SV2 vector (4 μg) with or without expression vector encoding GAP or GAP-C (1 μg). The GAP expression vector was constructed by inserting the complete human GAP cDNA sequence into the SV2 vector. The same transcriptional regulatory sequences were used to drive the expression of the cDNA sequence encoding GAP-C (nucleotides 2104 to 3140 of the GAP cDNA). Columns a through g show the effect of GAP or GAP-C on the transactivation due to SV2 vector without insert (a) or expression of polyoma middle T (b), v-Src (c), SDC25-C (d), wild-type Ras (e), oncogenic Ras-Val^{12} (f), or v-Mos (g). Data are expressed as described in Fig. 1.

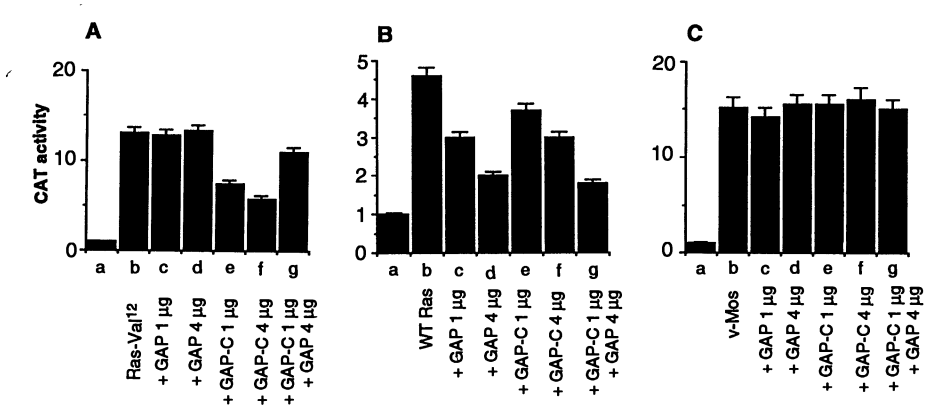

Fig. 3. Competitive effects of GAP and GAP-C in the presence of oncogenic Ras. CHO cells were cotransfected, as described in Fig. 1, with Py-Tk-CAT (0.5 μg) and 1 μg (c) or 4 μg (d) of vector encoding GAP, 1 μg (e) or 4 μg (f) of vector encoding GAP-C, or a combination of the vectors encoding GAP-C (1 μg) and GAP (4 μg) (g). Columns b through g show CAT activity from cells also transfected with vector (0.1 μg) encoding oncogenic Ras-Val^{12} (**A**), wild-type Ras (**B**), or v-Mos (**C**). Wild-type Ras or 0.1 μg of v-Mos were added respectively. Data are expressed as in Fig. 1.

that an effector-signaling domain is present in the NH_2-terminus of GAP. Such a model is also supported by studies showing that the inhibition muscarinic atrial K^+ channel currents by Ras depends on interaction of Ras with GAP (*17*, *22*). The putative signaling domain on GAP seems to be located within the sequence containing Src-homology regions because experiments done with a truncated form of GAP lacking the NH_2-terminal hydrophobic region (nucleotides 392 to 3140) gave results in our experiments identical to those obtained with full-length GAP (*15*). However, expression of a fragment of GAP containing amino acids 1 to 701, which contains the SH2-SH3 domain but lacks the binding region to Ras, did not modify the transactivation of Py-TK-CAT in presence or absence of any of the plasmids described above (*15*). Thus it is not clear which domain on the NH_2-terminus of GAP is critical for signal transduction. Increased expression of GAP in CHO cells did not modify signaling by oncogenic Ras. It may be that GAP, as an effector, is already more abundant than Ras (*23*) or that the supply of GAP that acts as an effector is tightly regulated within the cell.

GAP may be a direct link between Ras-GTP and other downstream effectors. GAP enhances the action of transforming v-Ha-Ras and insulin in *Xenopus* oocytes (*24*), although GAP itself is unable to promote a full maturation response in oocytes (*25*). These data indicate that GAP may act to transmit signals to other effectors, although it is possible that GAP enhances the Ras effect by binding an inhibitor of maturation, such as a related *rap* gene product. One interpretation of our data is that GAP-C, lacking SH2-SH3 sequences and the regions necessary to bind lipids, recognizes and traps the oncogenic Ras-GTP complex and thereby prevents it from properly eliciting a downstream signal. This model also implies that GAP-C should induce phenotypic reversion. However, GAP-C does not have anti-oncogenic properties (*12*, *14*), perhaps because the affinity of GAP-C for Ras-GTP is not high enough. It is not known whether the relative or absolute amount of Ras-GTP is more important for signaling by Ras. However, a modified GAP-C with higher affinity for Ras, such as that displayed by NF1-GAP (an alternative effector for Ras-GTP in all cells) (*26*, *28*) might induce reversion of oncogenic Ras transformation. For example, Rap1a, which competes with GAP for binding to Ras in vitro (*29*, *30*), has anti-oncogenic properties in a cell line transformed by v-Ki-*ras* (*31*). Blocking the interaction between Ras-GTP and GAP is thus a potential mechanism of action for a therapeutic drug.

Note added in proof: The SH2-SH3 domain of GAP appears to participate in the inhibition of muscarinic atrial K^+ channel currents by Ras (*32*).

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Oncogenic Forms of p53 Inhibit p53-Regulated Gene Expression

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Mutant forms of the gene encoding the tumor suppressor p53 are found in numerous human malignancies, but the physiologic function of p53 and the effects of mutations on this function are unknown. The p53 protein binds DNA in a sequence-specific manner and thus may regulate gene transcription. Cotransfection experiments showed that wild-type p53 activated the expression of genes adjacent to a p53 DNA binding site. The level of activation correlated with DNA binding in vitro. Oncogenic forms of p53 lost this activity. Moreover, all mutants inhibited the activity of coexpressed wild-type p53, providing a basis for the selection of such mutants during tumorigenesis.

The gene for the nuclear phosphoprotein p53 is the most commonly mutated gene yet identified in human cancers (*1*). Missense mutations occur in tumors of the colon, lung, breast, ovary, bladder, and several other organs (*2*–*4*). The *p53* gene can suppress the growth of transformed murine (*5*) or human cells (*6*, *7*), but oncogenic forms lose this suppressor function.

Evidence suggests that p53 may regulate gene transcription. A nuclear protein, p53 can bind to DNA in vitro both sequence specifically (*8*, *9*) and nonspecifically (*10*). In addition, the NH_2-terminus of p53 behaves as an acidic transcriptional activation domain when fused to GAL4 (*11*, *12*).

In this report we test whether p53 can directly activate gene expression through its DNA binding site and whether oncogenic forms of p53 can interfere with this activation. Reporter plasmids (PG_n-CAT series) contained the polyomavirus early promoter and the chloramphenicol acetyl transferase (CAT) gene located downstream of a DNA sequence (PG) that binds p53 in vitro (Fig. 1). The reporter and an expression vector that encoded human wild-type p53 (*13*) were transfected together into the human colorectal cancer cell line HCT 116 (*14*). This cell line makes low amounts of p53 protein (*15*).

The intact wild-type p53 protein acti-

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