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31. We thank J. Field for advice on the epitope addition method, T. Vernet for advice on site-directed mutagenesis, D Dignard for sequencing, and K. Matsumoto, S.-H. Kim, and D. Thomas for stimulating discussions. Supported by NIH grant CA 41996 (F.T); GM 35827 and a grant from the Lucille P. Markey Charitable Trust (J.R.); a NSF predoctoral fellowship (W.R.S.); and NIH Molecular and Cellular Biology Training grant GM 07183 (A.A.F.). F.T. is an Established Investigator of the American Heart Association. Lovastatin was a gift from A. Alberts (Merck, Sharp & Dohme Research Laboratories). The *gpa1*^{ts} allele was a gift from G. Cole and the *gpa1* disruption was a gift from K. Matsumoto. Antibodies to 3-phosphoglycerate kinase and to Sec61p were gifts from J. Thorner and from C. Stirling and R. Schekman, respectively. 12CA5 antibody was provided by I. Wilson. This is National Research Council of Canada publication number 31158.

9 January 1990; accepted 26 April 1990

Inhibition of GTPase Activating Protein Stimulation of Ras-p21 GTPase by the K*rev*-1 Gene Product

MATTHIAS FRECH, JACOB JOHN, VERONIQUE PIZON, PIERRE CHARDIN, ARMAND TAVITIAN, ROBIN CLARK, FRANK MCCORMICK, ALFRED WITTINGHOFER*

K*rev*-1 is known to suppress transformation by *ras*. However, the mechanism of the suppression is unclear. The protein product of K*rev*-1, Rap1A-p21, is identical to Ras-p21 proteins in the region where interaction with guanosine triphosphatase (GTPase) activating protein (GAP) is believed to occur. Therefore, the ability of GAP to interact with Rap1A-p21 was tested. Rap1A-p21 was not activated by GAP but bound tightly to GAP and was an effective competitive inhibitor of GAP-mediated Ras-GTPase activity. Binding of GAP to Rap1A-p21 was strictly guanosine triphosphate (GTP)–dependent. The ability of Rap1A-p21 to bind tightly to GAP may account for K*rev*-1 suppression of transformation by *ras*. This may occur by preventing interaction of GAP with Ras-p21 or with other cellular proteins necessary for GAP-mediated Ras GTPase activity.

GTPASE ACTIVATING PROTEIN (GAP) (*1, 2*) has been implicated as an effector of *ras* action by the following observations: (i) antibody to Ras-p21 inhibits *ras* function and prevents GAP interaction (*1*); (ii) GAP interacts with Ras-p21 at the effector binding site (*3–5*); and (iii) Ras-p21 mutants that bind tightly to GAP but cannot localize in the cell membrane inhibit the function of membrane-associated Ras proteins. This inhibition is relieved by addition of GAP (*6*).

The K*rev*-1 gene, also called *rap*1A, suppresses transformation of cells by the K*ras* oncogene (*7–9*). The predicted protein product is 53% identical to the K*ras* gene product, and contains all the sequences typical of a guanine nucleotide binding protein (*10*). Furthermore, the putative effector binding sequence of Ras proteins (a region essential for biological activity of *ras* oncogenes) is conserved in the K*rev*-1 gene product, Rap1A-p21. Thus, we tested the hypothesis that Rap1A-p21 may interact with the same effector as the Ras proteins and may suppress the *ras* transformation function by competing with Ras-p21 for that effector molecule.

We purified from *Escherichia coli* intact, recombinant Rap1A-p21 (60% purity) as well as a COOH-terminal truncated version of the protein that contained amino acids 1 to 168 (>95% pure). Like other small guanine nucleotide–binding proteins, Rap1A-p21 contains 1 mol of tightly bound nucleotide [GTP or guanosine diphosphate (GDP)], which is released slowly in the presence and rapidly in the absence of Mg^{2+} with kinetics similar to those of Ras-p21. The intrinsic GTPase activity (*11*) of Rap1A-p21 (4×10^{-3} min^{-1} at 37°C, Fig. 4, insert) was slow compared to that of Ras-p21 (2.8×10^{-2} min^{-1}) (*12*). Rap1A-p21 GTPase activity was not stimulated by addition of GAP (Fig. 1). Increasing the GAP: Rap1A-p21 molar ratio to 0.16 did not stimulate the GTPase activity. Under the same conditions, the GTP complex of Ras-p21 was hydrolyzed in 3 min.

Because oncogenic mutant forms of Ras protein that are not sensitive to GTPase stimulation by GAP nonetheless bind to GAP (*5*), we tested the ability of Rap1A-p21 to bind GAP. Affinity of binding was

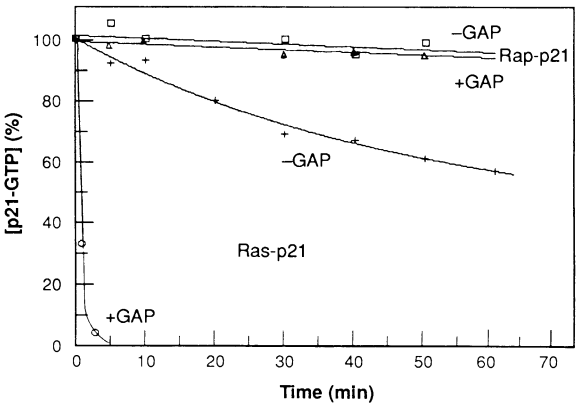

Fig. 1. Activation of the GTPase reaction of Ras-p21 and Rap1A-p21 by GAP. Normal and truncated (1–168) Rap1A-p21 were expressed in *Escherichia coli* with the ptacRas-expression system described for Ras-p21 (*21*). All proteins and the mutant (T61Q) (Fig. 4) were purified with the two-column procedure described (*12, 21*). Purity of Ras-p21, >95%; Rap1A-p21; ~60%; Rap1A (1–166), >95%; Rap1A(T61Q), 60%. Complexes between Ras-p21 or Rap1A-p21 and [γ-^{32}P]GTP were formed by incubating the proteins and radioactive nucleotide with 1 mM EDTA and 64 mM tris-HCl, pH 7.5, 1 mM dithioerythritol (DTE). Excess nucleotide was removed by gel filtration on a G-25 commercial column (NAP-5, Pharmacia LKB). The protein was eluted from GAP reaction buffer: 20 mM Hepes, pH 7.5, 1 mM DTE. The GTPase reaction was started by addition of 2 mM $MgCl_2$ and where indicated, the appropriate amount of recombinant human GAP (80 nM). Human GAP was expressed in sf9 insect cells (*22*) and was purified with a conventional S-Sepharose column and high-performance liquid chromatography (HPLC) on Mono-Q resin. The GTPase activity was measured as described (*2, 3, 5*) by following the decrease in concentration of [γ-^{32}P]GTP bound to p21 by filtration of the reaction mixture through nitrocellulose filters (0.45 μm). The data are plotted as the percentage of total Ras-p21-GTP converted to Ras-p21-GDP with time. The 100% value corresponds to 500 nM Ras-p21 or Rap1A-p21 (28,000 cpm).

M. Frech, J. John, A. Wittinghofer, Max-Planck-Institute für medizinische Forschung, Abteilung Biophysik, Jahnstrasse 29, 6900 Heidelberg, Germany.
V. Pizon, P. Chardin, A. Tavitian, INSERM U248, Faculté de Medecine Lariboisière Saint-Louis, 75010 Paris, France.
R. Clark and F. McCormick, Department of Molecular Biology, Cetus Corporation, Emeryville, CA 94608.

*To whom correspondence should be addressed.

measured by the ability of Rap1A-p21 to inhibit the effect of GAP on Ras-p21 GTPase activity. Rap1A-p21 in its GTP-bound form was a powerful inhibitor of GAP-mediated Ras-p21 GTPase (Fig. 2). The GTPase-stimulating activity of GAP was completely inhibited at 2 μM Rap1A-p21. The inhibition data were fitted to hyperbolic curves and gave an apparent inhibition constant (K_i) of 49 ± 5 nM, as determined with various concentrations of Ras-p21 and GAP (*13*). Inhibition of the interaction between Ras-p21 and GAP was strictly dependent on Rap1A-p21 being in the GTP-bound form. Rap1A-p21 in the GDP-bound conformation did not inhibit the GAP-mediated GTPase activity of Ras-p21 even at 1 μM (Fig. 2). Therefore, the affinity of the Rap1A-p21–GDP complex for GAP is at least 100 times less than that for the Rap1A-p21–GTP complex.

The dissociation constant (K_D) for bovine GAP and wild-type Ras-p21–GTP has been estimated to be about 110 μM (*5*). Because the affinity of Rap1A-p21 for GAP measured here was 2000 times greater than estimates of the affinity of Ras-p21 for GAP, we reevaluated these latter estimates. We determined the inhibition constants of Ras-p21 bound to the nonhydrolyzable GTP analogs, GppCp and GppNp with nonsaturating substrate (Ras-p21, [γ-^{32}P]GTP) concentrations. Competitive inhibition was assumed, thus the apparent K_i values were considered to be dissociation constants. Inhibition of the GAP–Ras-p21 reaction with increasing concentrations of the inhibitors gave apparent K_D values of 2.3 ± 0.25 μM for Ras-p21.GppCp and 4.8 ± 0.90 μM for Ras-p21.GppNp (Fig. 3). These affinities are much higher than those described for GAP from bovine brain (*5*).

The three-dimensional structure of Ras-p21 in the active GTP conformation reveals that the effector region and the region composed of amino acids 61 to 65 are located close to each other and both regions point into the solvent (*14*). Hras-p21 and Rap1A-p21 show 57% identity over the first 166 residues, with many conservative replacements (*7, 8*). We built a model of Rap1A-p21 with the coordinates of the Hras-p21.GppNp complex and replaced the divergent amino acids (*15*). Two amino acids were added to the polypeptide chain (Glu after residue 123 in loop L8 and Cys after residue 137 in loop L9) (*14*), and the structure was minimized with the Powell routine of the program package Quanta (Polygen) and XPLOR (*16*). No unallowed contacts or other high-energy terms were left in the structure. Indeed, the predicted structure of Rap1A-p21 was very similar to that of Ras-p21 and differed significantly only in the region of amino acids 61 to 65.

On the basis of sequence similarity to the *Drosophila ras*-related gene, D*ras*-3, three *rap* genes have been isolated (*rap*1A, *rap*1B, and *rap*2) (*8*). Whereas all the Ras-related proteins have Gln^{61} conserved, the Rap-p21 proteins have a threonine at position 61. Gln-61 is near the γ-phosphate in the three-dimensional structure of the Ras-p21. GppNp complex and may be involved in GTP hydrolysis (*17*). Thr^{61} of Rap-p21 was mutated to Gln and the mutant protein (T61Q) was isolated. The rate constant for the in vitro rate of GTP hydrolysis by Rap1A-p21 (T61Q) was 2 × 10^{-2} min^{-1} (37°C, Fig. 4, insert), and is about five times as great as that of wild-type Rap1A-p21. In addition, this rate of hydrolysis is about as fast as that of Ras-p21 (2.8 × 10^{-2} min^{-1}). This supports the notion that Gln^{61} is in-

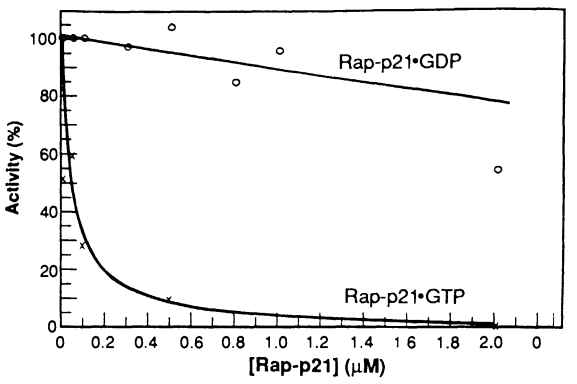

Fig. 2. Inhibition of Ras-p21-GAP interaction by Rap1A-p21. GTPase activity was measured as in Fig. 1 in the presence of increasing concentrations of Rap1A-p21 isolated from *E. coli*. HPLC analysis indicated that it contained GDP tightly bound to the protein. Rap1A-p21 was converted into the triphosphate conformation with unlabeled GTP as described in Fig. 1. The data are plotted as fractional activity, which is $V_{obs} - V_o/V_{max} - V_o$ versus Rap1A-p21 concentration. V_{max} is the maximal GAP-stimulated GTPase activity under these conditions and V_o is the GTPase activity in the absence of GAP.

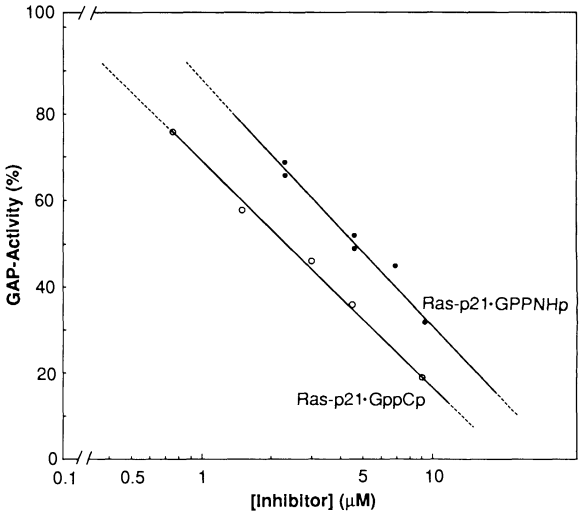

Fig. 3. Inhibition of Ras-p21 interaction with GAP by complexes of Ras-p21 with GppNp and GppCp. The reaction conditions are as in Fig. 1, in the presence of increasing concentrations of the inhibitors, and the data are plotted as residual activity as described in Fig. 2.

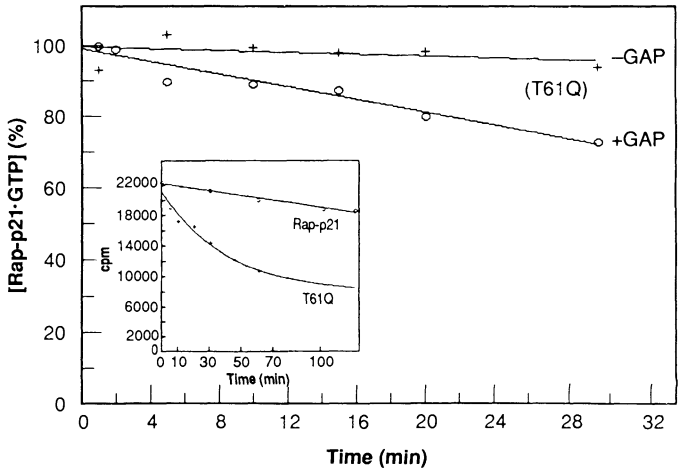

Fig. 4. In vitro GAP-stimulated GTPase activity of Rap1A-p21 with the mutation T61Q under standard GAP assay (*2, 3, 5*) conditions at 25°C. The insert shows the intrinsic GTPase activity (without GAP) of wild-type and mutant (T61Q) Rap1A-p21 at 37°C.

deed involved in GTP hydrolysis (*17*). Rap1A-p21 (T61Q), unlike wild-type Rap1A-p21, is slightly activated by GAP (Fig. 4), although the GAP-activated GTP hydrolysis is still much slower than that of Ras-p21 (Fig. 1). In addition, the affinity of Rap1A-p21 (T61Q) for GAP was greatly decreased (K_i = 1 ± 0.20 μm).

In summary, we have found that the protein product of the K*rev*-1 gene, Rap1A-p21, acts as a potent inhibitor of GAP-mediated activation of Ras-p21 GTPase activity. In platelets (*18*) the concentration of Rap1A-p21 is at least ten times as high as that of Ras-p21, suggesting that, in vivo, rap1A-p21 might limit the amount of GAP available for interaction with Ras-p21. Because GAP may be necessary for *ras* action, K*rev*-1 may thus suppress transformation of cells by *ras* oncogenes. Conversely, the amount of GAP available for binding to Rap1A-p21 may be limited in cells expressing high concentrations of oncogenic Ras proteins that have high affinities for GAP (*5*).

Interaction between GAP and Rap1A-p21 was shown here to be GTP-dependent, as is the interaction of GAP with Ras-p21. However, association of Rap1A-p21 with GTP is likely to be controlled, in part, by its own GTPase activating protein (smg-p21-GAP, Rap-GAP) (*19*). Biological activity of Ras-p21 may therefore be modulated directly by competition for GAP and indirectly by the effect of Rap-GAP on Rap1A-p21.

We propose the following model for GAP function. First, GAP binding to Ras-p21.GTP may cause a conformational change in Ras that is an intermediate in the GTP hydrolysis reaction (*20*). This change likely involves amino acids around position 61 of ras-p21, because this region appears to be involved in GTP hydrolysis (*17*). According to basic thermodynamic considerations, GAP itself is expected to undergo a conformational change upon binding. This form of GAP ("GAP*") then perhaps serves as the effector of Ras-p21 action. GAP* may also interact with other effector molecules, or would itself generate second-messenger molecules. Hydrolysis of GTP by Ras-p21 terminates the GAP* state. When GAP binds to Rap1A-p21.GTP, these conformational changes do not occur, the GAP* state is not achieved, and activation of GTP hydrolysis cannot take place.

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23. We thank D. Sanders and R. Goody for helpful discussions; G. Petsko for help with model-building of rap1A-p21; E. O'Rourke, D. Lowe, and K. Holmes for technical support.

26 January 1990; accepted 1 June 1990

Activation of Polyphosphoinositide Hydrolysis in T Cells by H-2 Alloantigen But Not MLS Determinants

ANNE M. O'ROURKE, MATTHEW F. MESCHER,* SUSAN R. WEBB

Murine minor lymphocyte-stimulating (Mls) determinants are cell surface antigens that stimulate strong primary T cell responses; the responding T cells display restricted T cell receptor (TCR) V_β gene usage. Interaction of T cells with mitogens or major histocompatibility complex (MHC) antigens activated the polyphosphoinositide (PI) signaling pathway, but this pathway was not triggered by Mls recognition. However, interleukin-2 (IL-2) secretion and proliferation to all three stimuli were comparable. Thus, although recognition of both allo–H-2 and Mls determinants is thought to be mediated by the TCR, these antigens appear to elicit biochemically distinct signal transduction pathways.

T CELLS RESPOND TO ALLOGENEIC MHC proteins and to foreign antigen in the context of self-MHC proteins. Products of the murine *Mls* loci stimulate primary T cell responses in an MHC class II–dependent, but unrestricted manner (*1–3*). The physical structure of Mls determinants is unknown; their presence is detectable only by virtue of T cell proliferative and secretory responses. T cell recognition of Mls^a determinants involves limited TCR V_β gene usage; for example, T cells bearing $V_\beta6$ and $V_\beta8.1$. TCR are selectively deleted in Mls^a mouse strains (*4, 5*). These and other data indicate that recognition of both Mls determinants and allo-MHC antigens involve TCR α/β heterodimers. The interaction of T cells with allogeneic MHC proteins (murine H-2) or foreign antigen and self H-2 molecules initiates the hydrolysis of T cell membrane PI with concomitant formation of the second messengers inositol phosphate and diacylglycerol (*6–8*). Studies with both cytotoxic and helper T lymphocytes have suggested the existence of additional, as yet undefined, TCR-coupled second messenger pathways important in generation of the functional responses to antigen (*9, 10*). By contrast, the signaling pathways coupling recognition of Mls to proliferation and secretion have not been examined.

Initial experiments that investigated transmembrane signaling by Mls^a-reactive T cells used $V_\beta6^+$ T cells purified from CBA/CA (*H-2*^{k}, *Mls*^{b}) mice, primed in vitro with Mls^a-positive CBA/J spleen cells (*H-2*^{k}, *Mls*$^{a/c}$) (*11*). These $V_\beta6^+$ T cells made secondary proliferative responses to Mls^a-bearing CBA/J and AKR/J (*H-2*^{k}, *Mls*^{a}) stimulators and to concanavalin A (Con A), but not to syngeneic CBA/Ca (*H-2*^{k}, *Mls*^{b}) cells (Fig. 1A). In parallel experiments, $V_\beta6^+$ T cells labeled with [^{3}H]myo-inositol (*12*) displayed a significant increase in [^{3}H]*PI turn-*

A. M. O'Rourke and M. F. Mescher, Division of Membrane Biology, Medical Biology Institute, La Jolla, CA 92037.
S. R. Webb, Department of Immunology, Scripps Clinic and Research Foundation, La Jolla, CA 92037.

*To whom correspondence should be addressed.