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19. The COOH-terminal region of the protein contains eight–amino acid–long repeats of proline, glutamic acid, serine, and threonine residues. Similar but not identical repeats exist in the β subunit of RNA polymerase II [L. Allison, J. Wong, V. Fitzpatrick, M. Moyle, C. Ingles, *Mol. Cell. Biol.* **8**, 321 (1988)]. This region of the 225 protein displays a high proline content (25%) and may function like the proline-rich activation region of CTF/NF-1 [N. Mermod, E. O'Neill, T. Kelly, R. Tjian, *Cell* **58**, 741 (1989)].
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21. Single letter abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
22. The nucleotide sequence of 225 has been submitted to GenBank and assigned the accession number M33672.
23. We thank A. S. Fauci for encouragement and support and for his critical reading of the manuscript. E. Tsachler and R. C. Gallo also generously provided RNA samples from several HTLV-I–transformed cell lines.

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In Vivo Receptor-Mediated Phosphorylation of a G Protein in *Dictyostelium*

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Extracellular adenosine 3′,5′-monophosphate (cAMP) serves multiple roles in *Dictyostelium* development, acting as a chemoattractant, a cell-cell signaling molecule, and an inducer of differentiation. The *Dictyostelium* G-protein α subunit Gα2 appears to be the major transducer linking the surface cAMP receptor to these intracellular responses. On stimulation of cells with cAMP, Gα2 is phosphorylated on one or more serine residues, resulting in an alteration of its electrophoretic mobility. Phosphorylation of Gα2 is triggered by increased occupancy of the surface cAMP receptor and is rapid and transient, coinciding with the time course of activation of physiological responses.

TRANSDUCTION OF A CAMP SIGNAL in *Dictyostelium* through the cell-surface cAMP receptor triggers multiple responses in the cells (*1*). The cAMP receptor contains seven transmembrane domains, a characteristic of G protein–linked receptors, such as rhodopsin and the β-adrenergic receptor (*2, 3*). A G-protein α subunit from *Dictyostelium*, Gα2, was determined to be the major transducer in this pathway by examination of the *Frigid A* complementation group (*4, 5*). These variants, which fail to carry out chemotaxis, increase intracellular cAMP or guanosine 3′,5′-monophosphate (cGMP) concentrations, or differentiate (*6*), contain point mutations (*7*) or deletions in the Gα2 gene (*5*). Because Gα2 is an essential component in the pathway, we investigated whether it undergoes covalent modification during cAMP stimulation.

To examine the Gα2 protein during the course of cAMP stimulation, we analyzed samples on immunoblots with a specific peptide antibody (*5*). Gα2 normally migrates as a 40-kD band during SDS–polyacrylamide gel electrophoresis (SDS-PAGE). However, Gα2 underwent a time-dependent transition in electrophoretic mobility after application of the cAMP stimulus (Fig. 1). The reduced mobility form of Gα2, with an apparent size of 43 kD, appeared after 20 s and peaked after 1 to 2 min. The transition in mobility was transient; the reduced mobility form returned almost completely to the original form within 15 min (Fig. 1A).

The following observations suggest that the cAMP-induced change in Gα2 mobility is induced by an increased occupancy of the surface cAMP receptor. (i) The mobility transition of Gα2 is a response that adapts. Many cAMP-induced responses, such as the activation of adenylyl and guanylyl cyclases, adapt after several minutes of constant exposure to cAMP (*8*). Removal of cAMP results in resensitization of the system (*9*). After 15 min in cAMP, Gα2 had returned predominantly to the prestimulus form (Fig. 1A). A second addition of the same amount of cAMP did not induce a second change in Gα2 mobility (Fig. 1B, lane 1), unless the cAMP stimulus was first removed and a recovery period allowed (Fig. 1B, lanes 2 and 3). (ii) The cAMP dose-response of the transition in Gα2 mobility is similar to other responses coupled to the cAMP receptor; alteration in Gα2 mobility is induced by nanomolar cAMP and saturated at 200 to 500 n*M* cAMP (*10, 11*). (iii) In cell lines transformed with cAMP receptor antisense sequences, which do not express cAMP receptors during development (*3*) but do express Gα2, there is no cAMP-induced alteration of Gα2 (Fig. 2).

The receptor-mediated transition in Gα2 electrophoretic mobility suggested that the protein undergoes a reversible, covalent modification, such as phosphorylation. We

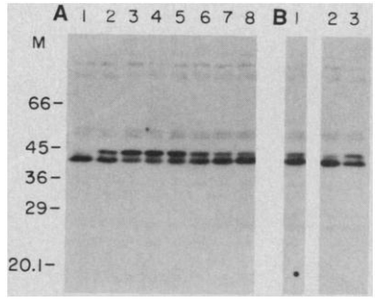

Fig. 1. Transient, receptor-mediated Gα2 modification. (**A**) Time course of Gα2 mobility shift in intact cells. Lanes 1 to 8 correspond to sampling times of 0, 20 s, 40 s, 1 min, 2 min, 5 min, 10 min, and 15 min after the addition of 1 μ*M* cAMP to basal state cells. (**B**) The effect of a second addition of cAMP. (Lane 1) Cells were exposed to 1 μ*M* cAMP for 15 min, after which a second addition of 1 μ*M* cAMP was made. The cells were sampled after a further 1 min. (Lanes 2 and 3) Cells were exposed to 1 μ*M* cAMP for 5 min then washed free of cAMP, placed into fresh medium, and incubated for 10 min without cAMP (lane 2) and then restimulated with 1 μ*M* cAMP for 1 min (lane 3). In both (A) and (B) cultures of 2×10^7 cells per milliliter were allowed to differentiate in DB (5 m*M* Na_2HPO_4, 5 m*M* KH_2PO_4, 2 m*M* $MgCl_2$, and 0.2 m*M* $CaCl_2$) as described (*18*). After 5 to 6 hours, cells were resuspended at a density of 5×10^7 cells per milliliter in DB containing 2 m*M* caffeine, which blocks the natural cAMP oscillations (*19*). Cells were shaken for 20 to 30 min to allow complete removal of endogenous cAMP and to allow the system to reach a basal state. Before the addition of cAMP, 10 m*M* dithiothreitol was added to inhibit extracellular phosphodiesterases (*20*). At the indicated times, cells were added to boiling SDS–sample buffer and subjected to SDS-PAGE as described (*21*), except that gels contained 0.033% SDS to improve separation of the Gα2 bands. Immunoblots were performed as follows: Gels were transferred to nitrocellulose (*22*), and after blockage of nonspecific sites with 3% bovine serum albumin (BSA), were incubated with antiserum to a peptide corresponding to the NH_2-terminal of Gα2 (*5*). Bands were visualized with ^{125}I-labeled protein A.

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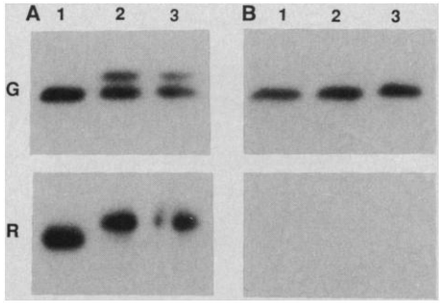

Fig. 2. Gα2 modification requires surface cAMP receptor. (Lanes 1 to 3) Immunoblot of cells incubated for 0, 1, and 5 min in 1 μ*M* cAMP. Receptor antisense (**B**) and vector control cell lines (**A**) have been described (*3*). Conditions of cell development and cAMP stimulation were as described in Fig. 1. Samples are a particulate cell fraction prepared by osmotic lysis with saturated ammonium sulfate as described (*23*). Blots were incubated in antiserum to either the Gα2 peptide (G) or the cAMP receptor (R). The observed change in receptor mobility in control cell lines, which occurs upon cAMP binding, and the absence of receptor in the antisense cell lines have been described (*3, 24*).

tested this possibility by incubating cells with ^{32}P-labeled inorganic phosphate ([^{32}P]P_i), followed by immunoprecipitation of Gα2. Phosphorylation of Gα2 was visible within 40 s, peaked after 1 to 2 min, and then disappeared during the next 15 min,

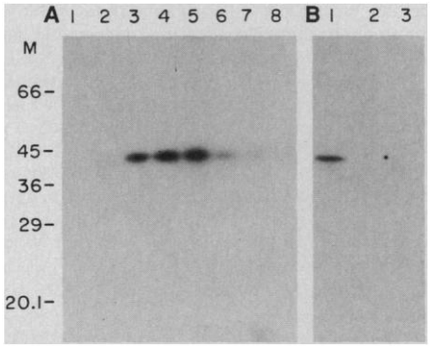

Fig. 3. Immunoprecipitation of ^{32}P-labeled Gα2. (**A**) Lanes 1 to 8 correspond to times of cAMP stimulation of 0, 20 s, 40 s, 1 min, 2 min, 5 min, 10 min, and 15 min. Cells were allowed to develop as in Fig. 1, except that phosphate was replaced with 20 m*M* Mes buffer, *p*H 6.2 [Mes-DB (*23*)]. After 5 to 6 hours of development, cells were resuspended in Mes-DB at a density of 10^8 cells per milliliter with 2 m*M* caffeine and [^{32}P]P_i (1 mCi/ml) for 30 min. A particulate cell fraction was prepared as in Fig. 2, except the final pellet was dissolved in SDS–sample buffer containing 0.5% SDS. Immunoprecipitation was performed as previously described with antiserum to Gα2 peptide (*23*). Immunoprecipitated samples were subjected to SDS-PAGE followed by autoradiography. (**B**) Immunoprecipitation controls of ^{32}P-labeled cells stimulated with 1 μ*M* cAMP for 1 min. Immunoprecipitation was performed with Gα2 antiserum (lane 1); preimmune serum (lane 2); and antiserum to Gα2 in the presence of 50 μg of the Gα2 peptide used to generate the antiserum (lane 3).

paralleling the transition in Gα2 mobility (Fig. 3A) (*12*). Immunoprecipitation was specific for Gα2; the radiolabeled band was not precipitated by preimmune serum nor by serum incubated with an excess of Gα2 peptide (Fig. 3B).

Radioactive phosphorus could be removed from immunoprecipitated Gα2 by treatment with alkaline phosphatase (Fig. 4A). The dephosphorylation caused a return to the increased mobility form of Gα2 (Fig. 4B), confirming that the transition in Gα2 mobility reflects its phosphorylation. The identity of the phosphorylated amino acid was determined by partial acid hydrolysis of the phosphorylated protein. Only phosphoserine and P_i appeared when the hydrolysates were subjected to electrophoresis on cellulose plates (Fig. 4C). No phosphoserine was detected when the same regions from unstimulated cells were examined. The phosphoserine residue is present within the first 119 amino acids of Gα2, since treatment of radiolabeled Gα2 with *N*-chlorosuccinimide, which cleaves after tryptophan (*13*), releases a radiolabeled peptide that migrates as an 18-kD band on SDS-PAGE and is immunoprecipitated by the NH_2-terminal antibody (*10*). This NH_2-terminal fragment contains 12 of the 28 serines present in Gα2; however, only 4 of the 12 are conserved among G-protein α subunits.

The receptor-mediated, in vivo phosphorylation of an essential G-protein α subunit is anticipated to have a significant function. Phosphorylation of Gα2 is a rapid, physiological response coupled to the excited state of the surface cAMP receptor. At least 1 mol of phosphate per α subunit is added, because the protein shifts its electrophoretic mobility. In vivo phosphorylation has been reported for $G_i\alpha$ in hepatocytes (*14*) and $G_z\alpha$ in platelets (*15*). The function of $G_z\alpha$ phosphorylation is unknown, whereas it was suggested that phosphorylation of $G_i\alpha$ was related to receptor desensitization in hepatocytes. In our experiments, the transient nature of the Gα2 phosphorylation during constant exposure to cAMP implies that it is related to activation, which subsides during continuous cAMP stimulation, rather than adaptation, which persists. The *Dictyostelium Frigid A* mutants serve as convenient hosts to assess the role of G-protein α-subunit phosphorylation. Transformation of the Gα2$^-$ cells with the wild-type Gα2 gene (*16*) restores Gα2 expression and its cAMP-induced phosphorylation (Fig. 5). Construction of strains that lack the phosphorylated serine residues will provide insight into the role of phosphorylation.

In current models of receptor–G protein–effector pathways (*17*), excited surface receptors catalyze the dissociation of guanine

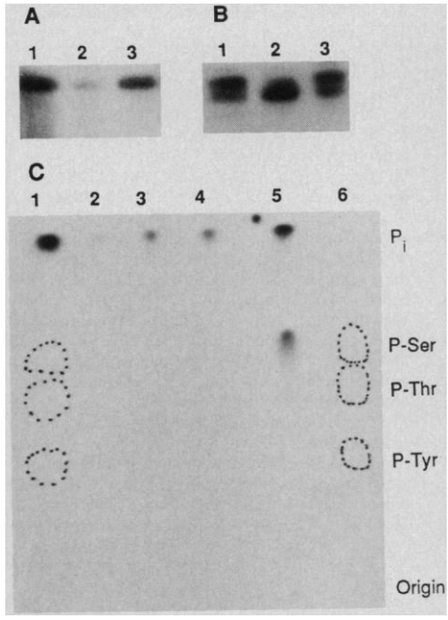

Fig. 4. (**A**) Alkaline phosphatase removal of ^{32}P from Gα2. (Lane 1) No alkaline phosphatase; (lane 2) plus alkaline phosphatase; (lane 3) plus alkaline phosphatase in the presence of 10 m*M* K_2HPO_4 (*p*H 7.5). Phosphorylated Gα2 bound to protein A–beads was incubated in 50 m*M* Hepes (*p*H 7.5), 0.05% Triton X-100, 25 m*M* KCl, and 15 m*M* MgCl, in the absence or presence of 5 U of calf intestine alkaline phosphatase at 37°C for 45 min before elution with SDS–sample buffer. Samples were subjected to SDS-PAGE and autoradiographed. (**B**) Immunoblot of alkaline phosphatase–treated Gα2. (Lanes 1 to 3) Alkaline phosphatase treatment of unlabeled Gα2 is as described in (A). The immunoblot was performed as in Fig. 1. (**C**) Phosphoamino acid determination of ^{32}P-labeled Gα2. Cells were labeled with [^{32}P]P_i as described in Fig. 3. Samples from unstimulated cells and cells that were stimulated for 1 min in 1 μ*M* cAMP were immunoprecipitated, subjected to SDS-PAGE, and transferred to nitrocellulose. Four areas were cut from the nitrocellulose after autoradiography: the area of unshifted Gα2 from the unstimulated (lane 2) and stimulated cells (lane 3); the area of phosphorylated Gα2 from unstimulated cells (lane 4); and phosphorylated Gα2 from stimulated cells (lane 5). The phosphoamino acid was identified after trypsin treatment of the nitrocellulose samples to release the label and subsequent incubation for 2 hours with 6*M* HCl at 110°C. Hydrolysates were dissolved in water containing the standards, phosphoserine, phosphothreonine, and phosphotyrosine and separated by electrophoresis at *p*H 3.5 as described (*24*). Standards were detected with 2% ninhydrin. For clarity the standard spots are shown only in lanes 1 and 6. Lane 1 also contains [^{32}P]P_i.

diphosphate (GDP) from an inactive G-protein αβγ heterotrimer, allowing the binding of guanine triphosphate (GTP) by the α subunit and dissociation of the heterotrimer into active α and βγ subunits. The activated subunits can then interact with effectors until hydrolysis of bound GTP by the α subunit and reassociation of the subunits. Thus, according to the model, regula-

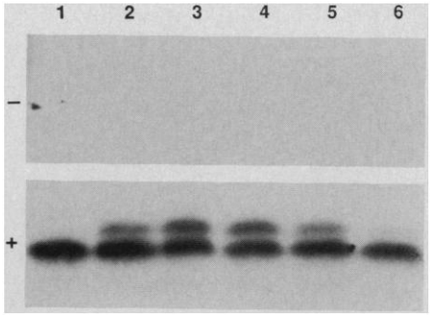

Fig. 5. Expression and phosphorylation of Gα2 from an extrachromosomal plasmid in a Gα2$^-$ cell line. Lanes 1 to 6 correspond to sampling times of 0, 30 s, 1 min, 2 min, 5 min, and 15 min after the addition of 1 μ*M* cAMP to basal state cells. The Gα2$^-$ cell line was generated by homologous recombination with a truncated Gα2 sequence. These cells were complemented by means of a full-length, wild-type Gα2 sequence inserted into an extrachromosomal vector (*16*). The cells were differentiated as described in Fig. 1, except that they were pulsed with 50 n*M* cAMP at 6-min intervals as previously reported (*19*). Immunoblots prepared as described in Fig. 2. The minus sign indicates the Gα2$^-$ cell line; the plus sign indicates the addition of the Gα2 plasmid.

tion occurs through conformational changes induced by GTP binding and hydrolysis. Our results suggest that, in vivo, there may exist an additional level of regulation of this pathway, α-subunit phosphorylation.

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Tick Anticoagulant Peptide (TAP) Is a Novel Inhibitor of Blood Coagulation Factor Xa

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A low molecular weight serine protease inhibitor (TAP) was purified from extracts of the soft tick, *Ornithodoros moubata*. The peptide is a slow, tight-binding inhibitor, specific for factor Xa (K_i = 0.588 ± 0.054 n*M*). The inhibitor also acts as an anticoagulant in several human plasma clotting assays in vitro. Its amino acid sequence (60 residues) has limited homology to the Kunitz-type inhibitors. However, unlike other inhibitors of this class, TAP inhibits only factor Xa. It had no effect at a 300-fold molar excess on factor VIIa, kallikrein, trypsin, chymotrypsin, thrombin, urokinase, plasmin, tissue plasminogen activator, elastase, or *Staphylococcus aureus* V8 protease. TAP's specificity and size suggest that it may have therapeutic value as an anticoagulant.

TICKS ARE HEMATOPHAGOUS ARthropods that must overcome host hemostasis in order to locate blood and maintain its flow during ingestion. Antihemostatic factors in the saliva of these and other blood-feeding organisms are probably directed toward platelet aggregation, coagulation, and vascular contraction. Although the mechanics and physiology of the blood-sucking process in ticks has been well studied (*1*), little is known about the sites of action or the molecular properties of the anticoagulant substances produced by these animals.

Inhibitors of factor Xa (*2, 3*) and thrombin (*4*) have been identified and partially purified from tick saliva. Tick saliva also blocks clotting via the intrinsic pathway (*5*), perhaps by inhibiting factor IXa (*4*). Antiplatelet activity that blocks platelet aggregation induced by adenosine diphosphate, collagen, or platelet-activating factor is also present (*5*).

To better define the anticoagulant activities present in ticks, a crude soluble extract of whole ticks was prepared and fractionated by gel filtration on Sephadex G-50. When selected column fractions were assayed, a peak with the ability to inhibit factor Xa (*6*) eluted with an apparent molecular size of 8,000 to 10,000. Peaks of both thrombin and platelet aggregation inhibitory activity were identified that were somewhat larger in size, 15,000 to 20,000. Trypsin inhibitory activity was also monitored, and was present throughout the column. Plasminogen activator activity was not detected in crude extracts or in column fractions.

The factor Xa inhibitory activity was purified by a combination of gel filtration, anion-exchange chromatography, and reversed-phase high-performance liquid chromatography (RP-HPLC). Most of the inhibitory activity eluted in one peak on RP-HPLC (Fig. 1A). This material was rechromatographed to obtain a homogeneous preparation of the protein (Fig 1B). We estimate that 200 to 250 μg of the purified tick anticoagulant peptide (TAP) could be obtained from 500 ticks. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) showed a single polypeptide with a molecular size of 6000 (Fig. 1C).

The purified TAP was used to characterize its inhibitory properties. The inhibition of factor Xa activity by TAP was concentration-dependent (Fig. 2). Preliminary evidence suggested that the inhibition of factor Xa was reversible and stoichiometric. An intrinsic inhibition constant K_i of 0.588 ± 0.054 n*M* was calculated by the Morrison method for tight-binding inhibi-

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