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GTP Binding by Class II Transactivator: Role in Nuclear Import

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Class II transactivator (CIITA) is a global transcriptional coactivator of human leukocyte antigen–D (HLA-D) genes. CIITA contains motifs similar to guanosine triphosphate (GTP)–binding proteins. This report shows that CIITA binds GTP, and mutations in these motifs decrease its GTP-binding and transactivation activity. Substitution of these motifs with analogous sequences from Ras restores CIITA function. CIITA exhibits little GTPase activity, yet mutations in CIITA that confer GTPase activity reduce transcriptional activity. GTP binding by CIITA correlates with nuclear import. Thus, unlike other GTP-binding proteins, CIITA is involved in transcriptional activation that uses GTP binding to facilitate its own nuclear import.

Class II transactivator (CIITA) is a transcription factor that controls the expression of multiple genes, including class II major histocompatibility complex (MHC), human leukocyte antigen–DM and invariant chain, involved in the exogenous pathway of antigen processing and presentation (*1*). Constitutive and inducible expression of class II MHC requires CIITA, as shown by analysis of immunodeficient patients who lack functional CIITA (*2*) as well as analysis of gene knockout mice (*3*). CIITA is a transcriptional coactivator in that, although it does not bind DNA per se, fusion of CIITA with the GAL4 DNA binding domain results in transactivation of promoters bearing the GAL4 target DNA (*4*). CIITA interacts with the MHC promoter binding factor RFX5, components of the basal transcription complex, including the transcription factor TFIIB and a subset of TATA-binding protein (TBP)–associated factors (TAFs), the transcription factors B cell octamer-binding protein (Bob1) and CREB-binding protein (CBP) (*5*).

Structural and mutational analyses have shown that CIITA contains acidic activation and proline-serine-threonine–rich (*6*) domains required for transcriptional activation (*1, 4, 7*). In addition, CIITA contains three putative GTP-binding protein motifs: a P-loop or Walker A motif [G1, consensus sequence $GX_4GK(S/T)$: ^{420}GKAGQGKS^{427}], a magnesium coordination site (G3, DXXG: ^{461}DAYG^{464}), and a site that confers specificity for guanosine [G4, (N/T/S)KXD: ^{558}SKAD^{561}] (*1, 7, 8*). A mutation in any of these three motifs reduces the transactivation function of CIITA (*7*). Hence, CIITA may represent a GTP-binding protein; however, the function and significance of these GTP-binding motifs have not been shown.

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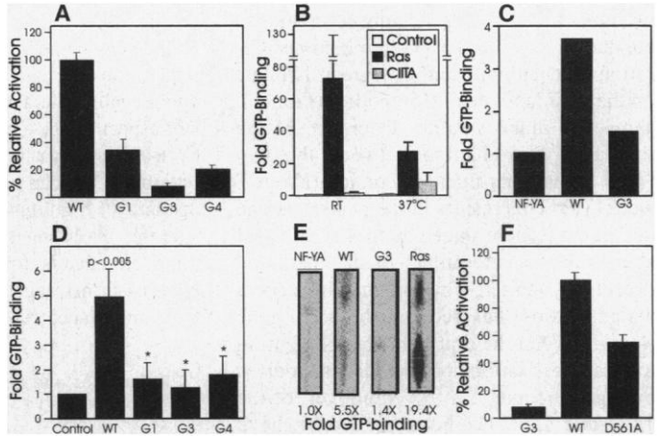

Fig. 1. Ability of CIITA and mutants to activate transcription correlates with GTP binding. **(A)** GTP-binding motif mutations decrease activation of transcription. CIITA-defective RJ2.2.5 cells were cotransfected with 1 μg of DNA encoding FLAG-tagged wild-type CIITA (WT) and CIITA mutants GTP1(ΔGK) (G1), GTP2(ΔDAYG) (G3), and GTP3(ΔSKAD) (G4) and 1 μg of the HLA-DR promoter/luciferase reporter DRA300luc (*24*). Luciferase activity was determined 24 hours after transfection. Results are expressed as percent relative luciferase activity compared with CIITA-induced activity (140-fold) over the reporter alone. Means of two independent experiments in triplicate (±SEM) are shown. **(B)** CIITA binds GTP. In vitro translated and immunoprecipitated CIITA and RasQ61L were tested for GTP binding at 25°C (RT) and 37°C (*31*). Fold increase of GTP-binding is shown and represents three independent determinations (±SEM). **(C)** GTP binding by CIITA is specific. CIITA (WT), the mutant G3, and the non–GTP-binding protein NF-YA were tested in the GTP-exchange assay as described above. Binding was corrected for protein expression; mean of two experiments is shown. **(D)** GTP-binding motif mutations decrease GTP binding. CIITA, G1, G3, and G4 were tested for their ability to bind GTP at 37°C as described above. GTP-binding activity was corrected for protein expression and is expressed as fold increase above control (±SEM) for three independent experiments. *t* -test values were calculated for each data set. *P* values: CIITA versus control (shown), $P < 0.005$; mutants versus CIITA (*), $P \leq 0.01$. CIITA averaged 32,800 ± 11,200 counts per minute (cpm) (about 9.4×10^{16} cpm per mol of CIITA assuming 50 ng of in vitro translated product per immunoprecipitation). **(E)** CIITA binds GTP in vivo. COS-7 cells were transfected with 1 μg of the indicated construct and labeled as described (*32*). Representative TLC results are shown for guanine nucleotides eluted from the indicated proteins. The mean fold GTP-binding above background is shown for three separate labeling experiments. **(F)** D^{561} is important for activation of transcription. RJ2.2.5 transfected with DRA300luc reporter and either CIITA or D561A as in (A). Activation with G3 is shown for comparison. Means of two independent experiments in triplicate (±SEM) are shown.

GTP-binding proteins are typically molecular switches that control various cellular functions by assuming a GTP-bound "on" state regulated by a GTPase activity that returns the protein to a guanosine diphosphate (GDP)-bound "off" state (*7*). Protein-specific GTPase-activating proteins (GAPs) and guanine-nucleotide exchange factors further regulate GTP-binding protein function by favoring the GDP- and GTP-bound forms, respectively (*9*). Whereas most GTP-binding proteins are regulated in this manner, some GTP-binding proteins (for example, RhoE, Rap1A, Rnd1) are constitutively bound to GTP. These proteins are constitutively activated and are generally deficient in GTPase activity (*10, 11*). Small GTP-binding proteins (for example, Ras, Ran, Rac, Rho, cdc42) are involved in proliferation, cell-cycle control, transport, and cell morphology (*12*). The heterotrimeric GTP-binding proteins (for example, $G_{\alpha i}$, $G_{\alpha s}$) mediate extracellular signals via serpentine receptors (*13*). Elongation factors EF-Tu and EF-G represent a third group of GTP-binding proteins that are involved in translation (*14*). Despite this diversity of GTP-regulated biological functions, a direct role for GTP-binding proteins in transcriptional regulation has not been described.

Mutations in CIITA at the putative G1, G3, and G4 motifs [GTP1(ΔGK), GTP2(ΔDAYG), and GTP3(ΔSKAD)] ablated activation of a class II MHC promoter (Fig. 1A). To determine whether CIITA is a GTP-binding protein, CIITA was in vitro translated, immunoprecipitated, and tested in a GTP exchange assay (Fig. 1B). A GTPase-defective form of H-Ras in which $Q^{61}{}_{15}$ mutated to L (Q61L) was used as a positive control for GTP-binding experiments. GTP-binding by CIITA was better at 37°C than at room temperature (RT), whereas GTP binding by Ras was reduced at the higher temperature. At RT, CIITA binds negligible quantities of GTP; however, at the more physiologically relevant 37°C, GTP binding by CIITA was one-third that of Ras. CIITA bound substantially more GTP than the non–GTP-binding protein (NF-YA) in exchange assays (Fig. 1C). CIITA also bound guanosine 5′-[γ-thio]triphosphate (GTPγS) (*15*). In in vitro assays, all three mutant forms of CIITA exhibited significantly reduced GTP-binding activity (Fig. 1D). As an in vivo correlate, CIITA bound orthophosphate-labeled GTP intracellularly (Fig. 1E), whereas, NF-YA and the G3 mutant did not [consistent with the in vitro data]. Finally, the aspartate residue in the G4 motif is conserved among GTP-binding proteins and is known to be critical for GTP binding by coordinating the guanine ring (*16–18*). The CIITA G4 motif mutant D561A, with an aspartate to alanine mutation, showed impaired activity when expressed in a CIITA defective B cell line, demonstrating the importance of aspartate at this position (Fig. 1F).

Under the same nucleotide-exchange conditions, CIITA failed to bind detectable amounts of adenosine triphosphate (ATP) (*19*). We also used a method for detecting ATP-binding proteins that relies on the adenosine derivative, 5′-*p*-fluorosulfonylbenzoyladenosine (FSBA). FSBA mimics the ATP/Mg^{2+} chelate and binds the ATP-binding site of many ATP-binding proteins (*20*). Bound FSBA can be detected by antibodies to FSBA in an immunoblot procedure. Whereas the catalytic domain of protein kinase α was readily labeled by FSBA, the adenosine derivative did not bind CIITA, even with a range of FSBA concentrations (*21*). ATP also failed to compete for GTP binding to CIITA in a limited number of competition assays (*22*). The absence of detectable ATP binding does not rule out the possibility that CIITA binds ATP, but it does suggest a stronger preference for GTP.

To further determine whether CIITA is a GTP-binding protein, we replaced portions of CIITA's GTP-binding motifs with similar regions from Ras. In the mutants C2RASG1, C2RASG3, and C2RASG4, the G1, G3, or G4 domain, respectively, of CIITA was replaced with analogous sequences from Ras. These mutants were essentially identical to CIITA in the activation of a class II DRA promoter (Fig. 2A). As a more stringent assay

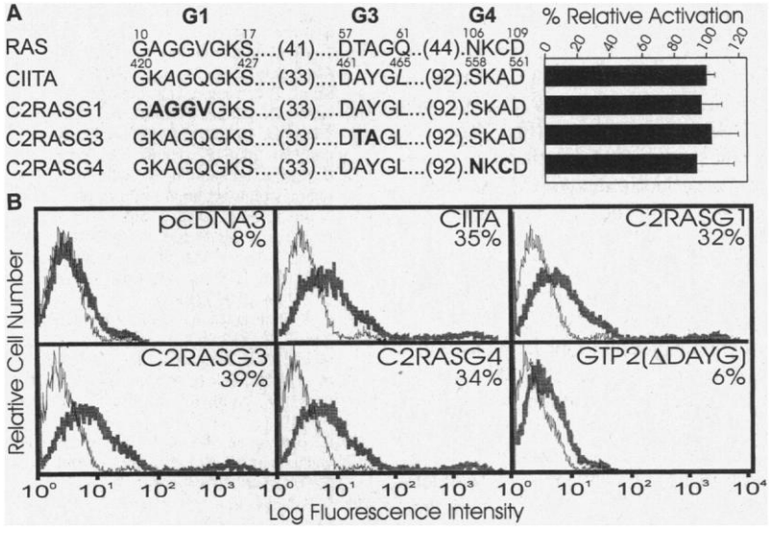

Fig. 2. Ras GTP-binding motifs substitute for the analogous domains in CIITA. (**A**) Sequence of nucleotide binding motifs G1, G3, and G4 from Ras, CIITA, and CIITA/Ras replacement mutants (C2RASG1, C2RASG3, and C2RASG4). Mutants were generated from p3FG.CIITA8 (a FLAG-tagged copy of the human CIITA cDNA in pcDNA3) (*7, 18*) by the transformer (Invitrogen) mutagenesis procedure as described by the manufacturer. RJ2.2.5 cells were transfected as described in Fig. 1A with 1 μg of the indicated constructs and 1 μg of DRA300luc, and luciferase activity was determined. Means of two independent experiments in triplicate (±SEM) are shown. (**B**) CIITA/Ras replacement mutants restore class II MHC expression. RJ2.2.5 cells were transiently transfected with the indicated constructs, selected with G418 (1 mg/ml) for 2 weeks, stained for surface expression of HLA-DR with monoclonal antibody L243 plus fluorescein isothiocyanate–coupled goat polyclonal antibody to mouse (GAM-FITC), and analyzed by fluorescence-activated cell sorting. Thin line, GAM-FITC alone; bold line, L243 and GAM-FITC; %, percentage of DR^+ cells in bulk culture with fluorescence intensities > 10^1.

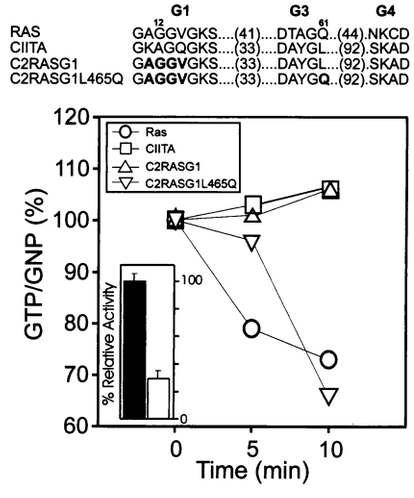

Fig. 3. GTPase activity can be conferred to CIITA. Ras and CIITA immunoprecipitates were prepared as in Fig. 1B and tested for GTPase activity at room temperature (Ras) or at 37°C (CIITA) as described (*31*). A decrease in bound GTP relative to all bound guanosine nucleotides, GTP + GDP + guanosine monophosphate [GTP/GNP (%)], indicates GTPase activity. Results are representative of three independent experiments; lower left inset is a transactivation assay, indicating percent relative DR promoter activation by wild-type CIITA (black bar) and C2RASG1L465Q (open bar) .

of function, we examined HLA-DR surface expression after transient transfection of the CIITA-Ras hybrids. Surface HLA-DR expression in bulk cultures was restored after expression of these constructs in CIITA-defective cells (Fig. 2B). Thus, sequences from Ras that are critical for binding to GTP are sufficient to substitute for their counterparts in CIITA in transcription activation. In contrast, the GTP-binding domain mutant GTP2(ΔDAYG) exhibited a markedly reduced ability to restore class II MHC expression (Fig. 2B).

Next, we tested whether CIITA exhibits GTPase activity. Examination of two residues within or near the individual motifs suggests that CIITA lacks appreciable GTPase activity. In the G1 motif of Ras, substitutions replacing G^{12} reduce intrinsic and GAP-induced Ras GTPase activity (*23*). The analogous residue in CIITA (422) is not a glycine but an alanine residue (Fig. 3). The Q61L mutation also drastically reduces the intrinsic and GAP-stimulated GTPase function of Ras, Ras-related proteins, and G_α subunits (*24*). The corresponding residue in CIITA (465) is a leucine (Fig. 3). Consistent with these observations, we did not detect a CIITA-associated GTPase activity (Fig. 3, □). The apparent lack of GTPase activity in CIITA suggests that CIITA is a constitutively active GTP-binding protein and that GTP may act solely to promote a functional conformation. Alternatively, CIITA may be an inefficient GTPase. It is also possible that there are CIITA-specific GAPs that could regulate the action of CIITA by inducing or increasing GTPase activity in CIITA.

The presence of A^{422} and L^{465} in CIITA is suggestive of a constitutively active GTP-binding protein. Substitution of the former with glycine and of the latter with glutamine might result in a CIITA molecule with GTPase activity. Similar mutations restored GTPase activity to the constitutively active and GTPase-deficient GTP-binding proteins RhoE and Rap1A (*9*). We generated the mutant C2RASG1L465Q, which possesses G^{422} and Q^{465}, by introducing a single amino acid mutation (L465Q) into C2RASG1 (Fig. 3), which provides the Ras GTPase-competent P-loop. This mutant displayed GTPase activity similar to that of wild-type H-Ras (Fig. 3, ∇). Wild-type CIITA and C2RASG1 did not exhibit GTPase activity. The ability of C2RASG1L465Q to activate the class II DRA promoter was impaired compared with CIITA (Fig. 3, inset). Mutation of L^{465} to Q alone without accompanying mutations in G1 also confers GTPase activity and is indistinguishable from C2RASG1L465Q in functional reporter assays. Thus, the loss of transactivation by CIITA correlates with the gain of CIITA GTPase activity and conversion to the GDP-bound state. This indicates that, like other GTP-binding proteins, the GTP-complexed state is the active form of CIITA.

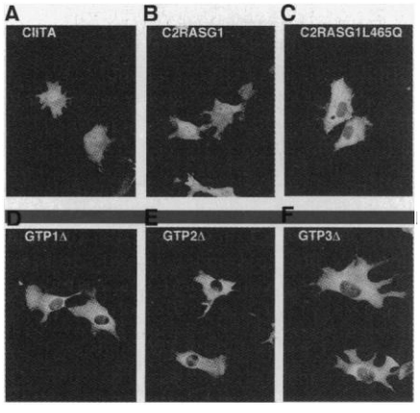

Fig. 4. Nuclear localization of CIITA correlates with GTP binding. COS-7 cells were transfected with 1 μg of the indicated construct and stained with anti-FLAG(M5) and GAM-FITC as described (*25*). GAM-FITC–stained control cells showed negligible staining.

As we have previously observed that CIITA is localized predominantly in the nucleus with limited cytoplasmic expression (*25*), the possible GTP dependence of CIITA nuclear import was examined (Fig. 4). CIITA mutants in the G1, G3, and G4 motifs fail to bind GTP and also fail to localize to the nucleus (Fig. 4, D to F). The CIITA-Ras hybrid C2RASG1 demonstrated nuclear localization similar to that of WT CIITA (Fig. 4, A and B), as did C2RASG3 and C2RASG4 consistent with their ability to activate transcription. A drastic decrease in CIITA nuclear localization was observed with the GTPase-positive mutant (Fig. 4C). The GTP-bound state of CIITA is therefore necessary for nuclear localization of CIITA, demonstrating that GTP-binding is important for at least this aspect of CIITA's coactivator function. These findings have been confirmed in two other cell lines, HeLa and G3A, which do not overexpress transfected CIITA (*26*).

These findings support the hypothesis that CIITA is a GTP-binding protein with the ability to directly regulate transcriptional activation and to mediate its own nuclear import. These results are compatible with the possibility that CIITA, like the GTPase-deficient Ras superfamily members RhoE and Rap1A, is constitutively GTP bound and active, whereas the GTP-free form is inactive. A GTP-dictated conformation could be required for several aspects of CIITA's function in addition to nuclear transport, such as stereospecific alignment with DNA-binding transcription factors, interaction with other proteins required for transactivation of class II MHC genes, and nuclear export.

A major remaining issue is how GTP regulates the function of CIITA in a biologic setting. The following are two potential scenarios in which GTP binding may be regulating the function. (i) GTP levels vary from cell type to cell type, with activated lymphocytes having abundant GTP (*27, 28*). Interestingly, activated lymphocytes also express more class II MHC antigens; it is plausible that the presence of enhanced MHC antigens is due to enhanced availability of GTP-bound CIITA. (ii) Nucleotide concentrations increase in proliferating lymphocytes (*27*). Class II MHC also increases as lymphocytes enter the cell cycle (*29*). Although this is not proof of regulation, these observations suggest possible conditions under which GTP availability could regulate CIITA.

GTP-binding proteins regulate a wide variety of cellular activities. Although ATP-dependent proteins have described roles in transcription (for example, Swi/Snf, GAGA) (*30*), CIITA is the only example of a GTP-dependent transcription factor. We speculate that this usage of GTP by CIITA indicates that CIITA may represent a family of GTP-dependent proteins functionally distinct from other GTP-binding proteins.

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Rapid Adaptation in Visual Cortex to the Structure of Images

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Complex cells in striate cortex of macaque showed a rapid pattern-specific adaptation. Adaptation made cells more sensitive to orientation change near the adapting orientation. It reduced correlations among the responses of populations of cells, thereby increasing the information transmitted by each action potential. These changes were brought about by brief exposures to stationary patterns, on the time scale of a single fixation. Thus, if successive fixations expose neurons' receptive fields to images with similar but not identical structure, adaptation will remove correlations and improve discriminability.

Figure 1 shows the response of a complex cell in striate cortex (V1) to a 0.5-s presentation of a stationary grating of optimal orientation, spatial frequency, phase, and size, followed by the same pattern presented as that of two brief probes of equal contrast, separated by 1.75 s (*1*). The initial response declined quickly. This decline, which is characteristic of cortical neurons (*2*), was more rapid than is seen in responses recorded at earlier stages in the visual pathway (*3*), so a considerable part of it must arise within cortex. The initial grating presentation also left the neuron desensitized, as can be seen by comparing the responses to the first probe (sensitivity was low) and to the second (sensitivity had recovered) (*4*). Desensitization does not result from light adaptation to the stationary image: First, a 0.5-s presentation of a grating flickering at 4 Hz was as effective an adapter as a stationary one (*5*); second, were light adaptation the cause of the changes, we would expect an increased response to a probe grating of the opposite spatial phase to the adapting grating. In fact, in complex cells, adaptation almost equally reduced responses to probes of either polarity.

Sensitivity changes in cortical neurons have generally been studied after prolonged periods of adapting stimulation (*6*), although evidently brief stimuli can quickly bring about significant reductions (Fig. 1) (*7*). Contrast adaptation, known to originate in cortex (*8*), is thought to adjust the responsivity of a neuron to the prevailing levels of contrast in the image (*9*). This prevents saturation of responses to strong stimuli and maintains the

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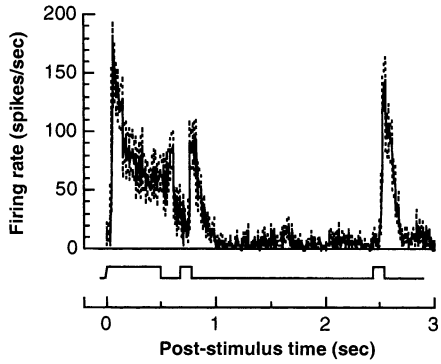

Fig. 1. Responses of a complex cell in V1 to presentations of a stationary sinusoidal grating (100% contrast) of optimal size, position, orientation, and spatial phase. The neuron was not directionally selective. The solid trace shows the mean discharge rate (computed from 40 stimulus presentations, in 10-ms bins), and the dotted traces show the mean ± 1 SEM. The lower trace identifies the times of onset and offset of the grating. The first (0.5 s) presentation of the grating desensitizes the cell, diminishing its response to the probe presented 200 ms later. By the time of the second probe, almost 2 s later, sensitivity has recovered. The time-constant of recovery of sensitivity measured by probing at a range of times (not all shown), was 8 s.