Modulation of cytokine-induced HIV gene expression by competitive binding of transcription factors to the coactivator p300

Michael O.Hottiger, Lisa K.Felzien and Gary J.Nabel[1]

Howard Hughes Medical Institute, University of Michigan Medical Center, Departments of Internal Medicine and Biological Chemistry, 1150 W.Medical Center Drive, 4520 MSRB I, Ann Arbor, MI 48109-0650, USA

[1]Corresponding author
e-mail: gnabel@umich.edu

The host response to viral infection involves the secretion of multiple cytokines which alter immune function and viral replication. These proteins activate several signal transduction pathways in infected cells which must be integrated to regulate cellular and viral gene expression. In this report, we demonstrate that specific transcription factors induced by distinct cytokines regulate HIV transcription by competitive binding to the p300 coactivator. Interferon-α (IFN-α) was found to inhibit NF-κB-dependent HIV gene expression stimulated by tumor necrosis factor-α (TNF-α). This inhibition was mediated by binding of the IFN-α signal transducer and activator of transcription 2, Stat2, to a specific domain of p300 which also binds to the RelA (p65) subunit of NF-κB. p300 was found to be limiting with respect to RelA (p65) and Stat2, and this effect was reversed by overexpression of p300. Inhibition by Stat2 was specific for NF-κB and was not mediated by Stat1, which is also induced by IFN-α. Gene activation induced by the Stat2 transcription domain was also inhibited by expression of RelA. These results demonstrate that HIV transcription can be regulated in the nucleus by competitive binding of specific cytokine-induced transcription factors to a discrete domain of a transcriptional coactivator.
Keywords: HIV/interferon-α/NF-κB/Stat2/TNF-α

Introduction

The activation of gene expression by cytokines results in activation of transcription factors which exert diverse effects on cellular genes (for reviews see Hill and Treisman, 1995; Marshall, 1995). The cytokines interferon-α (IFN-α) and tumor necrosis factor-α (TNF-α) represent mediators often released during immune and inflammatory responses that stimulate different patterns of cellular and viral gene expression. IFN-α inhibits the replication of many viruses (Dorr, 1993), including the human immunodeficiency virus (HIV) (Yamada *et al*., 1988; Fan *et al*., 1993; Bourinbaiar *et al*., 1995), for example, while TNF-α enhances HIV-1 transcription through induction of NF-κB which binds to two κB sites within the long terminal repeat (LTR) (Nabel and Baltimore, 1987). Recently, NF-κB transcriptional activity

was shown to be regulated by the activity of cyclin-dependent kinases (Cdks) through the p300 or the CREB-binding protein (CBP) coactivators (Perkins *et al*., 1997). The binding of the RelA (p65) subunit of NF-κB to p300/CBP was found to be mediated through the N-terminal region of p300, while cyclin E–Cdk2 interacted with the C-terminus. CBP was identified originally in a screening approach as a protein which interacted strongly with CREB (Chrivia *et al*., 1993; Kwok *et al*., 1994), and p300, which is highly related to CBP, was found based on its interaction with E1A (Eckner *et al*., 1994).

Both molecules are large nuclear phosphoproteins that appear to be involved in the regulation of cellular growth control (Eckner, 1996) and were also proposed to be critical for integration and regulation of different pathways, such as the inhibitory actions of the glucocorticoid receptor and the retinoic acid receptor on AP-1 activation (Kamei *et al*., 1996) or the nuclear signaling of the JAK/STAT and the Ras/AP-1 pathway (Horvai *et al*., 1997). It was reported recently that p300/CBP not only interacts with a histone acetyltransferase (P/CAF; Yang *et al*., 1996) but also itself possesses an intrinsic histone acetyltransferase activity (Bannister and Kouzarides, 1996; Ogryzko *et al*., 1996) and may therefore not only be an adaptor between DNA-binding factors and P/CAF but may also contribute directly to transcriptional regulation via targeted acetylation of chromatin. Interestingly, p300/CBP appears to be a molecule that is a point of conversion for viral proteins as well as for cellular signal transduction pathways (Nakajima *et al*., 1996; Torchia *et al*., 1997). In addition, p300 regulates a variety of transcription factors unrelated to NF-κB, including p53 (Lee *et al*., 1995; Gu *et al*., 1997; Lill *et al*., 1997a) Myb (Dai *et al*., 1996; Oelgeschlager *et al*., 1996), Jun (Bannister *et al*., 1995), Fos (Bannister and Kouzarides, 1995), Sap1a (Janknecht and Nordheim, 1996), Stat1 (Zhang *et al*., 1996), Stat2 (Bhattacharya *et al*., 1996), myogenic transcription factors (Eckner, 1996; Yuan *et al*., 1996) and nuclear receptors (Kamei *et al*., 1996; for reviews, see Eckner, 1996; Janknecht and Hunter, 1996; Shikama *et al*., 1997). Among these factors, the Stat2 protein induced by IFN-α as part of the ISGF-3 complex (Schindler and Darnell, 1995) binds to an N-terminal region of p300 in proximity to the RelA site that is distinct from that bound by other factors such as CREB, c-Jun, c-Myb, SAP-1 and YY1 (Bhattacharya *et al*., 1996; Eckner, 1996). This observation raised the possibility of competitive interactions among specific transcription factors to regulate gene expression induced by multiple signal transduction pathways, such as the inhibitory activity of IFN-α on the TNF-α-induced transcriptional activation of the HIV-1 LTR. In support of this possibility, we demonstrate that RelA and Stat2 can compete directly for p300 binding and, remarkably, that this competition dramatically influences transcriptional
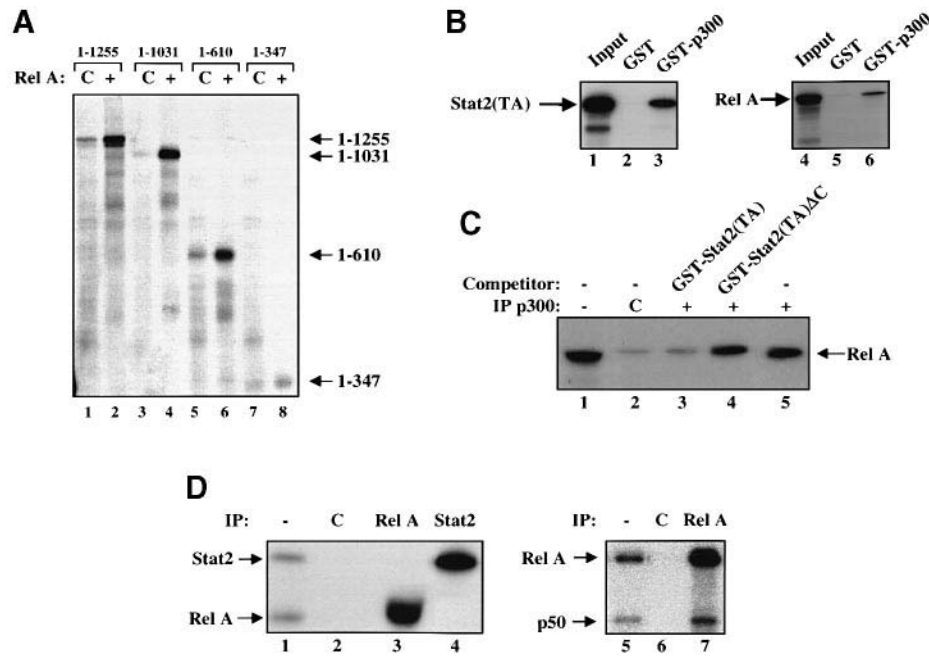
Fig. 1. Common binding domain and competition of RelA and Stat2 binding to p300. (**A**) RelA binds p300 between amino acid residues 1 and 610. RelA was immunoprecipitated from nuclear extracts of 293 cells which express transfected RelA and incubated with radiolabeled *in vitro* translated N-terminal deletion mutants of p300 (lanes 2, 4, 6 and 8, designated +). After three washes, bound proteins were resolved by SDS–PAGE. A non-specific rabbit IgG was included as a negative control (lanes 1, 3, 5 and 7, designated C). (**B**) RelA and Stat2 bind to the CH1 region on p300. Approximately 100 ng of GST–p300 (CH1) fusion protein was pre-bound to glutathione-conjugated agarose beads (lanes 3 and 6) and incubated with the radiolabeled *in vitro* translated transactivation domain of Stat2 (amino acids 670–851, left panel) or RelA (right panel). Purified GST was included as control (lanes 2 and 5). After washing, bound protein was resolved by SDS–PAGE. The lane designated 'Input' represents 10% of the input protein. (**C**) Competitive binding of Stat2 and RelA to p300 (CH1). Proteins from Jurkat nuclear extracts were immunoprecipitated with a control antibody (lane 2) or an antibody to p300 (lanes 3–5) and incubated with *in vitro* translated full-length RelA in the presence of GST–Stat2(TA) (lane 3) or a GST–Stat2(TA)ΔC control (lane 4). After washing, bound complexes were resolved by SDS–PAGE. Lane 1 contains 10% of the input protein. (**D**) RelA and Stat2 do not interact directly. *In vitro* co-translated RelA and Stat2 were immunoprecipitated with a control rabbit IgG (lane 2), an anti-RelA IgG (lane 3) or an anti-Stat2 IgG (lane 4). Under the same conditions, *in vitro* co-translated RelA and p50 were immunoprecipitated with a control rabbit IgG (lane 6) and anti-RelA IgG (lane 7). The lanes designated 1 and 5 represent 10% of the *in vitro* translated input protein.

activation of the HIV-1 LTR. Through this mechanism, p300 may regulate viral gene expression in the setting of simultaneously activated signal transduction pathways.

Results

Binding of RelA and Stat2 to the p300 CH1 domain and competition studies

To examine possible regulatory mechanisms that coordinate diverse signal transduction pathways, we investigated whether Stat2 and RelA might bind to the same region of p300. Incubation of 5′ deletion mutants of p300 with immunoprecipitated RelA revealed a region of p300 between amino acids 1 and 610 that was responsible for RelA binding (Figure 1A). Similar results were obtained for the transcription factor Stat2 (data not shown). This fragment contains a cysteine–histidine-rich region of p300, the CH1 domain (amino acids 302–442). An overlapping region (amino acids 300–528) has been shown previously to bind to Stat2 (Bhattacharya *et al.*, 1996). To localize the binding region for these proteins further, a GST–CH1 fusion protein (amino acids 302–422) was prepared and incubated with *in vitro* translated RelA or the transactivation domain of Stat2 (amino acids 670–851). This CH1 domain was necessary and sufficient for specific binding of Stat2 or RelA to p300 (Figure 1B), though additional non-specific binding to other regions of p300 or CBP cannot be excluded.

To determine whether the Stat2 and RelA transcriptional activation domains could bind competitively to this region of p300, competition experiments were performed. GST–Stat2(TA), which contains the transactivation domain of Stat2 (amino acids 670–851) fused to GST, reduced the binding of RelA to p300 to background levels compared with a control GST–Stat2(TA)ΔC fusion protein which lacked the C-terminal 83 amino acids (Figure 1C). This finding indicated that Stat2 competes with RelA for binding to p300 and that the C-terminal 83 amino acids which mediate binding of Stat2 to p300 (Bhattacharya *et al.*, 1996) are required for this competition. To exclude further the possibility that Stat2 could interact directly with RelA and thereby inhibit RelA binding to p300, we performed *in vitro* immunoprecipitations using the same buffer conditions as for Figure 1C. *In vitro* translated RelA and Stat2 did not co-associate in immunoprecipitation assays, indicating that RelA and Stat2 do not interact directly, whereas RelA under the same conditions interacted with p50 to form the expected heterodimer (Figure 1D).

Inhibition of TNF-α-stimulated NF-κB transcription by IFN-α

To determine whether endogenous Stat2 induced by IFN-α can inhibit TNF-α induction of endogenous NF-κB and its subsequent gene activation through this regulatory mechanism in the nucleus, cytokine stimulation studies
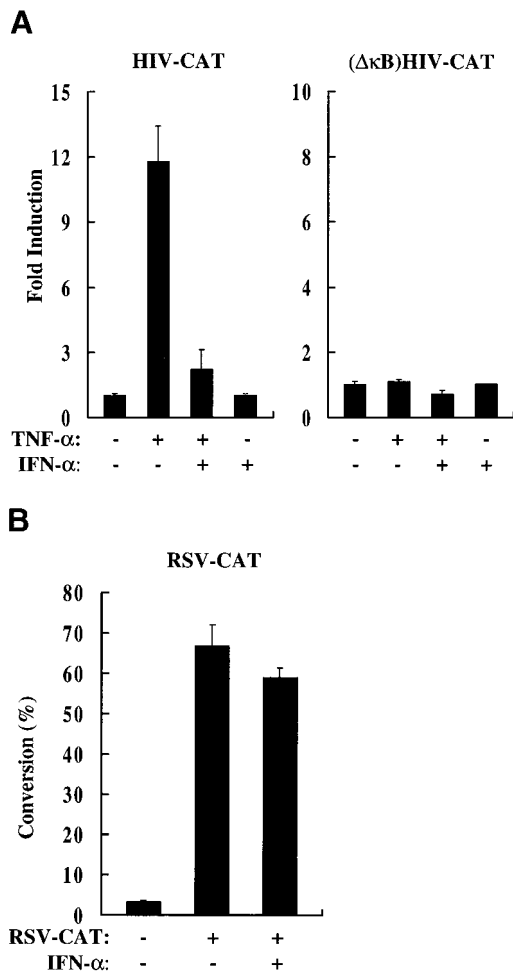
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A

B

Fig. 2. IFN-α inhibits TNF-α-induced HIV-1 gene expression in a κB-dependent manner. The specificity of IFN-α inhibition for a κB-dependent reporter. Jurkat T-leukemia cells were transfected with μg of HIV CAT or 5 μg of ΔκB-HIV CAT (**A**) or RSV–CAT (**B**) and pre-stimulated with 1000 U/ml IFN-α for 20 h followed by TNF-α stimulation (20 U/ml) for 4 h in (A) or stimulated for 20 h with 1000 U/ml IFN-α in (B). Error bars indicate SEM of three independent experiments.

were performed. Jurkat T-leukemia cells were transfected with an HIV–CAT reporter and incubated with IFN-α before adding a constant amount of TNF-α 20 h later. IFN-α inhibited κB-dependent CAT activity stimulated by TNF-α (Figure 2A), whereas no transcriptional activation of HIV–CAT was observed by IFN-α itself. No inhibitory effect of IFN-α could be observed in transfection assays with the relevant control (mutant κB) reporter plasmid, indicating that the observed inhibitory effect was dependent on NF-κB (Figure 2A). Identical treatment of Jurkat cells transfected with a Rous sarcoma virus (RSV)–CAT plasmid, which expresses CAT constitutively and independently of NF-κB, showed no difference in CAT activity (Figure 2B), indicating that this effect was specific for NF-κB and did not affect the activity of basal transcription factors.

IFN-α but not IFN-γ is able to inhibit RelA transactivation: lack of Stat1 involvement

IFN-α not only modifies Stat2 but also induces nuclear translocation of Stat1 as part of the ISGF-3 complex
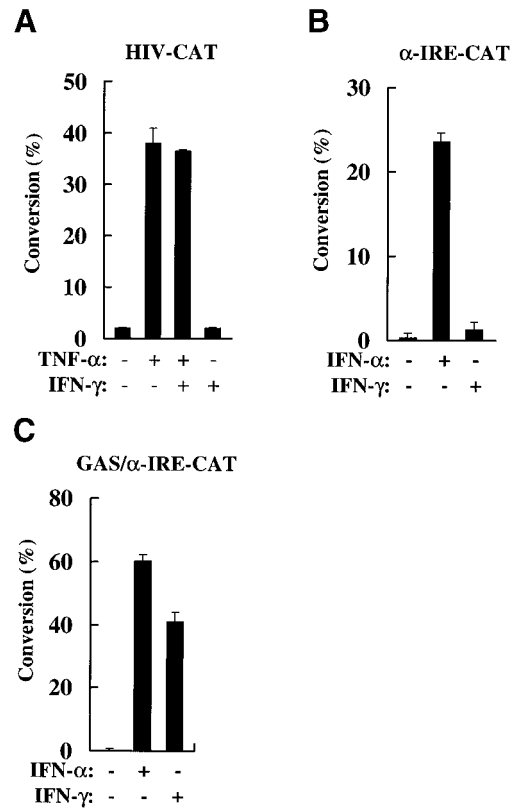
A

B

C

Fig. 3. IFN-γ does not inhibit TNF-α-induced HIV-1 gene expression. (**A**) IFN-γ does not affect κB-dependent gene activation. Jurkat T-leukemia cells were transfected with HIV–CAT (5 μg) and pre-stimulated with 1000 U/ml of IFN-γ for 20 h followed by TNF-α stimulation (20 U/ml) for 4 h. Error bars indicate SEM of three independent experiments. (**B** and **C**) Specific transcriptional activation by IFN-α and IFN-γ on different reporter plasmids. Jurkat T-leukemia cells were transfected with α-IRE-CAT (5 μg; B) or GAS/α-IRE–CAT (5 μg; C) reporter plasmid and subsequently stimulated with 100 U/ml of IFN-α or IFN-γ for 20 h. Error bars indicate SEM of three independent experiments.

(Schindler and Darnell, 1995). Stat1 has been shown to interact with p300, but its potential effects on RelA-mediated transaction through p300 have not been defined. To address whether Stat1 may affect p300-dependent NF-κB transcriptional activation, cells were stimulated with IFN-γ which causes nuclear translocation of Stat1 but not Stat2 (Schindler and Darnell, 1995), and its effect on TNF-α-induced NF-κB activity was determined. IFN-γ did not inhibit κB-dependent transcription stimulated by TNF-α, and no transcriptional activation of HIV–CAT was observed by IFN-γ itself (Figure 3A). This result excluded the possibility that Stat1 served as the competing molecule induced by IFN-α, and further suggested that Stat2 was required for competition with NF-κB. The presence of a functional IFN-γ receptor and Stat1 in Jurkat cells was confirmed with two different CAT reporter plasmids under the transcriptional control of different elements, a gamma-activated sequence (GAS) element, recognized by the Stat1 homodimer induced by IFN-γ, or an α-interferon-responsive element (α-IRE) regulated by the ISGF-3 trimer complex induced by IFN-α. α-IRE–CAT activity could be stimulated only by IFN-α, whereas the plasmid containing the α-IRE plus two GAS elements could be activated by either IFN-α or IFN-γ (Figure 3B
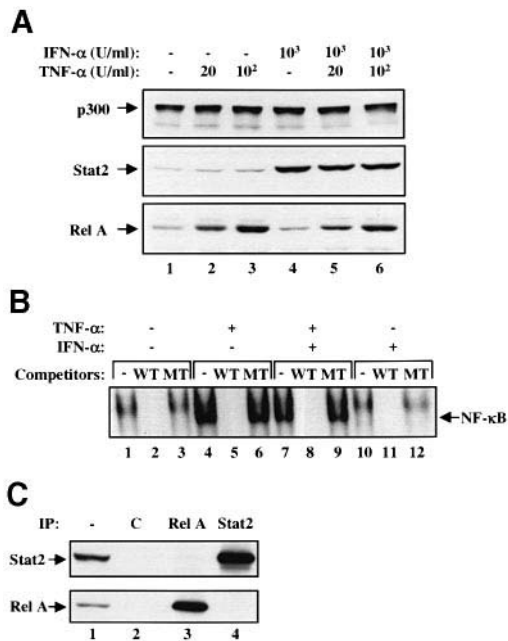
Fig. 4. Lack of IFN-α effect on nuclear translocation and induction of NF-κB DNA-binding activity, and RelA and Stat2 do not interact *in vivo*. (**A**) IFN-α has no effect on the nuclear translocation of the NF-κB subunit RelA. Western blot analysis of Jurkat T-leukemia cells which were unstimulated (lane 1), or stimulated with 20 U/ml TNF-α (lane 2) or 100 U/ml TNF-α for 4 h (lane 3), stimulated with 1000 U/ml IFN-α for 20 h (lanes 4), or stimulated with 1000 U/ml IFN-α for 20 h, followed by a stimulation with 20 U/ml TNF-α (lane 5) or 100 U/ml TNF-α for 4 h (lane 6). The upper panel shows the analysis for p300, the middle panel the analysis for Stat2, and the lower panel the analysis for RelA. For each lane, 30 μg of nuclear extracts were used. (**B**) IFN-α does not affect the NF-κB DNA-binding activity. EMSA of nuclear extracts from Jurkat cells unstimulated (lanes 1–3), stimulated with 1000 U/ml IFN-α for 20 h (lanes 10–12), stimulated with 20 U/ml TNF-α for 4 h (lanes 4–6) or stimulated with 1000 U/ml IFN-α, followed by a stimulation with 20 U/ml TNF-α for 4 h (lanes 7–9). (**C**) RelA and Stat2 do not interact *in vivo*. RelA induced by TNF-α (100 U/ml; for 4 h) and Stat2 induced by IFN-α (1000 U/ml; for 20 h) were immunoprecipitated with a control rabbit IgG (lane 2), an anti-RelA IgG (lane 3) or an anti-Stat2 IgG (lane 4). Lane 1 represents 20 μg of nuclear extract.

and C), thus confirming that both transcriptional activators were induced appropriately by IFN-α or IFN-γ in these cells, though only IFN-α, which induced Stat2, affected NF-κB/p300 binding.

Lack of IFN-α effect on the induction of RelA and NF-κB DNA-binding activity

TNF-α has been shown to induce NF-κB through its ability to activate the degradation of the inhibitory protein family IκB which sequesters NF-κB in the cytoplasm (Beg and Baldwin, 1993; Baeuerle and Henkel, 1994). To exclude the possibility that IFN-α could interfere with the TNF-α signal transduction pathway which causes degradation of IκB and liberation of NF-κB for nuclear import, we examined the amount of NF-κB translocated into the nucleus after TNF-α, or TNF-α plus IFN-α, treatment. Western blot analyses of nuclear RelA, Stat2, and p300 were performed (Figure 4A). The level of p300 was not affected by the treatment of cells with TNF-α or IFN-α, and the level of RelA was also unaffected by pre-treatment of cells with IFN-α. The electrophoretic mobility

shift assay (EMSA) was performed with nuclear extracts of the relevant cytokine-treated cells. This analysis revealed that NF-κB induction was not affected by pre-stimulation with IFN-α (Figure 4B), suggesting that TNF-α receptor function was intact in cells treated with IFN-α and that the mechanism of IFN-α inhibition of NF-κB did not involve degradation of IκB.

RelA and Stat2 do not interact in vivo

To exclude the possibility that Stat2 could interact with RelA *in vivo* and thereby influence the transcriptional activation by RelA, we performed an immunoprecipitation study. Immunoprecipitation of RelA or Stat2 from nuclear proteins after extraction of Jurkat cells stimulated with IFN-α and TNF-α showed no interaction between the two transcription factors (Figure 4C). This observation suggests that RelA and Stat2 interact with one another neither directly nor indirectly through p300. Analysis of competition between endogenous RelA and Stat2 for binding to p300 after cytokine treatment was not possible *in vivo* since the high salt concentration required for nuclear extract preparation disrupts the physiologic interactions of these proteins.

Inhibition of NF-κB or Stat2 transcription by the relevant counter-active cytokine or transcriptional activation domain

To confirm the physiological relevance of this interaction, we analyzed the effects of IFN-α and TNF-α, which induce the Stat2 and RelA transcription factors, respectively, through their relevant cellular cytokine receptors. Transfected RelA inhibited transcriptional activation of a CAT reporter containing a regulatory region from the 2′–5′ oligo adenylate synthetase (OAS) gene that is regulated by IFN-α (Figure 5A). Conversely, TNF-α activation of the HIV-1 enhancer by NF-κB was inhibited by an expression vector encoding a GAL4 fusion protein linked to the Stat2 transactivation domain [GAL4–Stat2(TA)], but not by GAL4–Stat2(TA)ΔC which lacked the C-terminal 83 amino acids (Figure 5B). When transfection of an expression vector encoding RelA was used to activate NF-κB gene expression, incubation with IFN-α, which induced Stat2 physiologically, exerted a similar inhibitory effect (Figure 5C). No inhibitory effect of GAL4–Stat2(TA) or IFN-α could be observed in transfection assays with the relevant control (mutant κB) reporter plasmid, indicating that the observed inhibitory effect was dependent on NF-κB (data not shown). Taken together, these results suggest that physiologically induced Stat2 can negatively regulate the transcriptional activity of RelA and vice versa, and that these effects are mediated through the transcriptional activation domains of each protein.

Competition by Stat2 and RelA for binding to p300 in vivo

The functional interaction of these proteins was demonstrated *in vivo* by transient transfection assays. Transfected RelA (Figure 6A) inhibited transcriptional activation by GAL4–Stat2(TA) on a GAL4 reporter plasmid. Conversely, co-transfections were performed with HIV–CAT and GAL4–Stat2(TA). GAL4–Stat2(TA) strongly inhibited HIV enhancer activation by transfected RelA, whereas GAL4–Stat2(TA)ΔC alone had no effect (Figure 6B). No
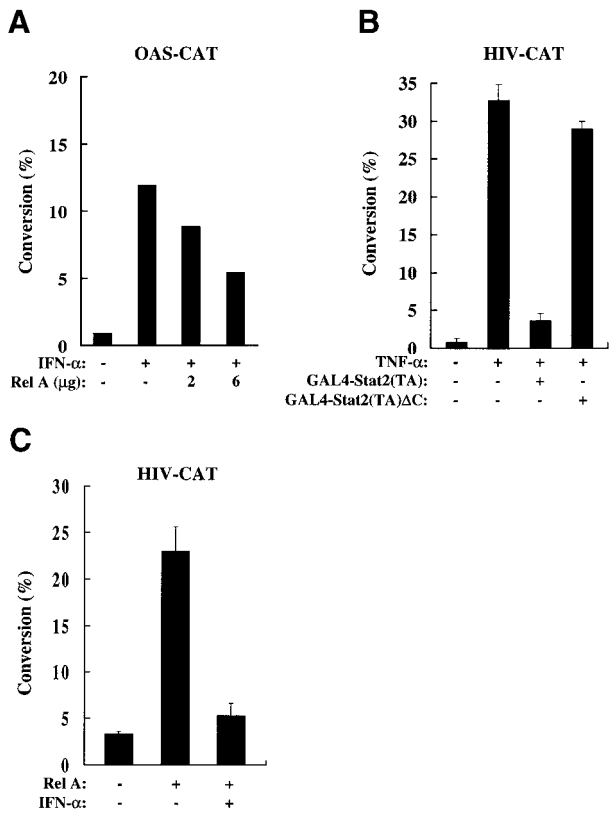
Fig. 5. Physiologically induced or transfected Stat2 or RelA cross-inhibit gene activation. (**A**) Transfection of an expression vector encoding RelA is sufficient to inhibit IFN-α-dependent gene activation. Jurkat cells were transfected with OAS–CAT (2 µg) and 2 or 6 µg of RelA expression plasmid. After 20 h, cells were stimulated with IFN-α (200 U/ml) for 16 h. A control vector with the same RSV regulatory region was included such that a total of 6 µg of RelA and control plasmids was present per sample. (**B**) Transfection of an expression vector encoding the transactivation domain of Stat2 is sufficient to inhibit RelA-dependent gene activation. Jurkat cells were transfected with HIV–CAT (5 µg) and GAL4–Stat2(TA) (2 µg) or GAL4–Stat2(TA)ΔC (2 µg) as indicated. After 20 h, cells were stimulated with TNF-α (20 U/ml) for 4 h. A control vector with the same RSV regulatory region was included such that a total of 2 µg of the GAL4–Stat2(TA) and control plasmids was present per sample. Error bars indicate SEM of three independent experiments. (**C**) Jurkat T-leukemia cells were transfected with 0.1 µg of RSV expression plasmids encoding RelA and HIV–CAT (5 µg). A control plasmid was included so that each sample contained a total of 0.4 µg of RSV expression plasmid. Cells were stimulated for 24 h with 1000 U/ml of IFN-α and harvested. Error bars indicate SEM of three independent experiments.

Fig. 6. The transactivation domain of Stat2 competes with the NF-κB subunit RelA for a limiting amount of p300 *in vivo*. (**A**) Gene activation by the transactivation domain of Stat2 is inhibited by transfection of a RelA expression vector. Jurkat cells were transfected with 2 µg of GAL4–CAT reporter plasmid, 0.2 µg of GAL4–Stat2(TA), and RelA as indicated. A control RSV vector was included such that the same amount of plasmids was present per sample. (**B**) Expression of transfected Stat2 represses RelA-mediated transcriptional activation. Jurkat cells were co-transfected with HIV–CAT (5 µg), GAL4–Stat2(TA) (2 µg) or GAL4–Stat2(TA)ΔC (2 µg), and RSV–RelA (0.2 µg) as indicated. A control RSV vector was included such that a total of 2 µg of expression plasmids was used in each sample. Error bars indicate SEM of three independent experiments. (**C**) Inhibition of transfected GAL4–Stat2(TA) by co-transfected RelA is relieved by expression of p300. Jurkat cells were transfected with HIV–CAT (2 µg), RSV–RelA (0.2 µg), GAL4–Stat2(TA) (2 µg) and CMV-p300 expression vector (5 µg) as indicated. A control CMV vector encoding alkaline phosphatase (AP) was included as a control such that a total of 5 µg of the p300 and control plasmids was present in each sample. Cells were harvested after 48 h. Error bars indicate SEM of three independent experiments. (**D**) Inhibition of transfected GAL4–Stat2(TA) by co-transfected RelA is relieved by expression of the CH1 domain of p300. Jurkat cells were transfected with HIV–CAT (2 µg), RSV–RelA (0.2 µg), GAL4–Stat2(TA) (2 µg) and different p300 deletion mutants (5 µg) as indicated. A control CMV vector encoding AP was included as a control such that a total of 5 µg of the p300 and control plasmids was present in each sample. Cells were harvested after 48 h. Error bars indicate SEM of three independent experiments. (**E**) Western blot analysis of RelA and GAL4–Stat2 from 293 cell nuclear extracts. 293 cells were transfected with 0.2 µg of GAL4–Stat2(TA) and 6 µg of RelA, and subsequently analyzed for GAL4–Stat2 (left panel) or transfected with RSV–RelA (0.2 µg) and GAL4–Stat2(TA) (2 µg) (middle panel) or RSV-RelA (0.2 µg), GAL4–Stat2(TA) (2 µg) and CMV-p300 expression vector (5 µg) (right panel) and analyzed for RelA. All transfections contained a LacZ reporter to standardize the loaded amount of proteins.

inhibition was seen with a control plasmid with mutated κB sites (data not shown). If competitive binding of Stat2 to p300 was responsible for this inhibitory effect rather than a general non-specific squelching effect which is particularly pronounced on unintegrated episomal reporter genes (Natesan *et al.*, 1997), it would be predicted that transfection of p300 should restore RelA-dependent activation in the presence of GAL4–Stat2(TA). Co-transfection of p300 with HIV–CAT, RelA and GAL4–Stat2(TA) indeed restored HIV enhancer activity 5-fold (Figure 6C). The comparison of the sample with RelA alone and RelA plus p300 did not show significant differences, in contrast to our previous study (Perkins *et al.*, 1997), because the amount of transfected p300 plasmid used in Figure 6C was suboptimal for trans-
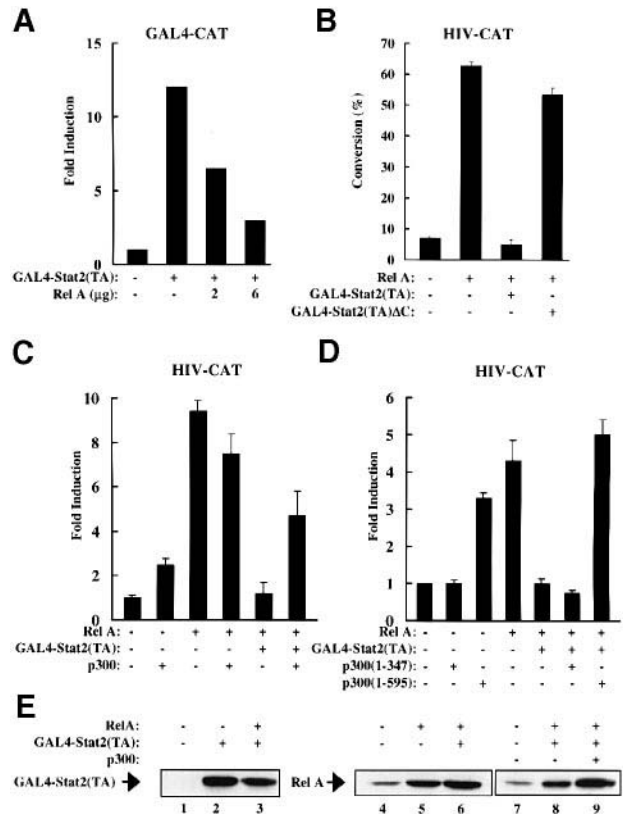
activation, thus simplifying the interpretation of the experiment. To confirm that the CH1 domain of p300 is limiting, another transfection experiment was performed with a

deletion mutant of p300 which contains the CH1 domain (amino acids 1–596) and a deletion mutant of p300 which lacks the CH1 domain (amino acids 1–349). The (amino acids 1–596) p300 mutant was able to restore the Stat2 repression in comparison with the mutant lacking CH1, indicating that the CH1 domain is indeed the domain responsible for the rescue of the repression *in vivo* (Figure 6D). This observation excludes the possibility that the Stat2 transactivation domain exerts a non-specific effect and is consistent with the hypothesis that Stat2 and RelA compete for binding to p300 *in vivo*. The CH1 p300 mutant showed some degree of activation by itself, suggesting that it may bind other endogenous transcription factors which can compete with RelA for binding to p300. In addition, this result suggests that the presence and total quantity of all p300/CBP family members in the cell nucleus is not sufficient to overcome the inhibitory activation of Stat2 after overexpression or physiological induction by IFN-α. To exclude further the possibility that the inhibitory effect of GAL4–Stat2 or RelA is due to interference with the expression level after transfection, we performed Western blot analysis with nuclear extract from cells transfected with the same DNA combinations as shown in Figure 6A–C. No significant difference in the expression level of RelA or GAL4–Stat2 could be detected (Figure 6D).

Inhibition of TNF-α-stimulated NF-κB transcription by IFN-α is dose dependent, and the time course of Stat2 induction and translocation by IFN-α

To determine whether IFN-α can inhibit TNF-α induction of endogenous NF-κB in a dose-dependent manner, cytokine stimulation studies were performed. Jurkat T-leukemia cells were transfected with an HIV–CAT reporter and incubated with increasing amounts of IFN-α before adding a constant amount of TNF-α 20 h later. IFN-α inhibited κB-dependent CAT activity stimulated by TNF-α in a dose-dependent manner (Figure 7A). To confirm that the IFN-α dose–response curve for inhibition of HIV–CAT expression correlates with the dose–response relationship for Stat2 activation, we performed an experiment using a reporter plasmid containing the CAT gene regulated by the α-IRE, which is activated by the Stat2-containing ISGF-3 complex specifically induced by IFN-α (Figure 7B). The activation of the α-IRE–CAT reporter correlated with the dose–response inhibition of HIV–CAT activity (Figure 7A). To determine whether the inhibitory activity of IFN-α on TNF-α-induced gene activation can be exerted on a stably integrated gene, we repeated the cytokine studies with the 1G5 cell line, which contains a stable integrated luciferase gene under the control of the HIV-1 LTR (Aguilar-Cordova *et al*., 1994). 1G5 cells were incubated with increasing amounts of IFN-α before adding a constant amount of TNF-α 20 h later. IFN-α inhibited κB-dependent luciferase activity stimulated by TNF-α in a dose-dependent and saturable manner (Figure 7C).

Both for transient transfection and stably integrated κB reporters, pre-incubation with IFN-α for at least 6 h was required for significant inhibition of TNF-α-stimulated NF-κB function (Figure 7D), indicating that cross-signaling of rapidly activated cellular protein kinases by these two pathways was unlikely. To understand the quantitative aspects of Stat2 induction by IFN-α, we determined the kinetics of Stat2 nuclear localization after IFN-α stimulation by exposure of Jurkat cells to this cytokine for different time intervals. Stat2 began to translocate to the nucleus shortly after stimulation with IFN-α (Figure 7E, 30 min). Longer incubation with IFN-α, however, increased the level of Stat2 significantly. The nuclear level of Stat2 increased up to 12 h after stimulation and remained at this level for at least 24 h (data not shown). This result is in agreement with previously published observations that Stat2 mRNA is induced upon IFN-α stimulation (Schindler *et al*., 1992). The quantitative increase in Stat2 and corresponding time of IFN-α incubation correlated with the requirement for pre-incubation to inhibit RelA transactivation (Figure 7A). We therefore conclude that pre-incubation with IFN-α was indeed necessary to accumulate sufficient nuclear Stat2 for competition with RelA.

p300 is limiting relative to cytokine-induced RelA and Stat2 in the nucleus

For competitive binding of RelA and Stat2 to occur *in vivo*, it would be necessary that these transcription factors be present in a high molar excess relative to p300/CBP family members. To determine whether p300 is limiting relative to these transcription factors, we quantitated the number of p300, RelA and Stat2 molecules per cell nucleus after appropriate cytokine stimulation. Immunoprecipitations were performed for p300 from unstimulated nuclear extracts, for RelA from TNF-α-stimulated extracts, or for Stat2 from IFN-α-stimulated extracts. Different dilutions of these immunoprecipitated proteins were then compared with a dilution of known protein standards with the same slope on silver-stained SDS–PAGE (Figure 8, legend) and with a defined number of cell equivalents of nuclear extract by Western blot analysis (Figure 8A–C). Immunofluorescence analysis suggested that the amounts of these transcription factors per cell is uniform in the cell population, and these proteins are found primarily in the nuclear fraction in the relevant cytokine-stimulated cells (data not shown). Thus, comparison of silver staining allowed an estimation of the number of molecules of each immunoprecipitated protein, and Western blot analysis of nuclear extracts from a specified number of cells provided a method to convert this quantity into the number of molecules per cell nucleus. No substantial difference was observed in the quantity of nuclear p300 in unstimulated or stimulated cells (Figure 4A). This quantification documented that p300 is limiting in the cell nucleus relative to either RelA or Stat2 after stimulation of cells with TNF-α or IFN-α, respectively (Figure 8). The observed difference is ~5-fold, and thus explains the inhibitory effect of cytokine-induced Stat2 on the transcriptional activation of NF-κB. Given the significant increase in levels of these transcriptional factors and apparent lack of increase in p300/CBP family members in response to cytokine treatment, it is likely that competition for a limiting amount of p300/CBP family members is responsible for this effect.

Discussion

Cellular activation by multiple cytokines leads to activation of multiple signal transduction pathways. The appropriate
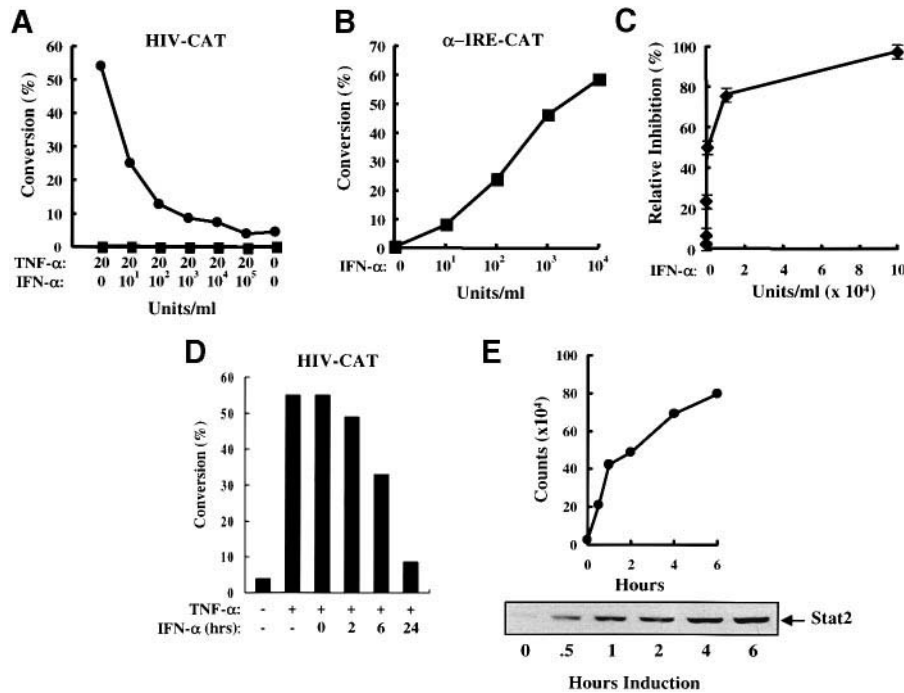
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Fig. 7. IFN-α inhibits TNF-α induced HIV-1 gene expression in a dose-dependent manner, and quantitation of Stat2 induction by IFN-α. (**A**) IFN-α inhibits κB-dependent gene activation in a dose-dependent manner. Jurkat T-leukemia cells were transfected with HIV–CAT (●, 5 μg) or ΔκB HIV–CAT (■, 5 μg) and pre-stimulated with the indicated amounts of IFN-α for 20 h followed by TNF-α stimulation (20 U/ml) for 4 h. (**B**) IFN-α activates the α-IRE–CAT in a dose-dependent manner. Jurkat T-leukemia cells were transfected with α-IRE–CAT (4 μg) and stimulated with the indicated amounts of IFN-α for 20 h. (**C**) 1G5 cells were pre-stimulated with the indicated amounts of IFN-α for 20 h followed by TNF-α stimulation (20 U/ml) for 4 h. Inhibition is defined relative to the level of inhibition observed with maximal concentrations of IFN-α (10^5 U/ml). Maximal IFN-α inhibition corresponded to a 50% inhibition of TNF-α-induced gene expression. Error bars indicate SEM of three independent experiments. (**D**) Time course analysis of IFN-α pre-treatment. Jurkat T-leukemia cells were transfected with HIV–CAT (5 μg) and pre-stimulated with 1000 U/ml IFN-α for the indicated times, followed by TNF-α stimulation (20 U/ml) for 4 h. (**E**) IFN-α induces nuclear translocation of Stat2 over 24 h. Jurkat cells (5.8×10^7) were simulated with 1000 U/ml IFN-α for different times as indicated and 20 μg of nuclear extract from each time interval was used for Western blot analysis of Stat2. Signals from the Western blot were quantitated using the ImageQuant program for the indicated time intervals.

coordination of these regulatory signals is essential both for viral replication and for cell function/survival. In this study, we show that TNF-α stimulation of HIV gene expression by NF-κB in Jurkat T-leukemia cells is inhibited by IFN-α but not by IFN-γ. The inhibition was specific for NF-κB, indicating that basal transcriptional regulatory proteins or other regions of the promoter were not involved. The inhibition occurred through a novel mechanism involving the competition for binding of two specific transcription factors, Stat2 and RelA, to the CH1 region of p300. A direct or indirect interaction between RelA and Stat2 was excluded by performing immunoprecipitations *in vitro* and *in vivo*. The identification of p300 as an integral component of the activation complex for IFN-α- and TNF-α-released transcription factors and the competition model for limiting amounts of p300 is strengthened further by the finding *in vivo* that overexpression of p300 was able to abolish Stat2 repression of RelA. The competition model was also supported by quantification of the relative molar amounts of p300, RelA and Stat2 in the nucleus after cytokine induction.

It has been known for some time and was recently reported that squelching effects caused by transactivator overexpression using transient transfection experiments can be misleading (Natesan *et al.*, 1997). To confirm the specificity of inhibition by transactivators in this present study, we analyzed these effects in three different ways. First, a reporter gene without the relevant *cis*-acting

regulatory sequences showed no inhibition in the presence of IFN-α-induced transcription factors or with co-transfected GAL4–Stat2 (Figures 2B, and 6B and D). Secondly, the inhibitory effects of Stat2 could be restored specifically by transfection of a vector encoding the full-length p300 coactivator or a deletion mutant expressing the CH1 domain (Figure 6C and D). Thirdly, these effects were confirmed using a stable integrated reporter gene under control of the same HIV regulatory region (Figure 7C). Finally, inhibition was seen with endogenous transcription factors induced by cytokines.

The possibility that p300/CBP could be a limiting factor for the integration of multiple signal transduction pathways had been suggested as a possible mechanism to explain inhibition of Ap1-dependent gene activation by steroid receptors and Stat1 (Kamei *et al.*, 1996; Horvai *et al.*, 1997), but quantitation and competitive binding have not been demonstrated previously. The competition between RelA and Stat2 for p300 binding supports this hypothesis and establishes that such a mechanism is used to regulate transcription, based on differential recruitment of p300- and CBP-containing coactivator complexes. Though it remains possible that TNF-α or IFN-α may modify p300 differentially and affect its ability to bind cytokine-induced transcription factors, the amount of p300 available for binding to the relevant transcription factor would remain rate limiting.
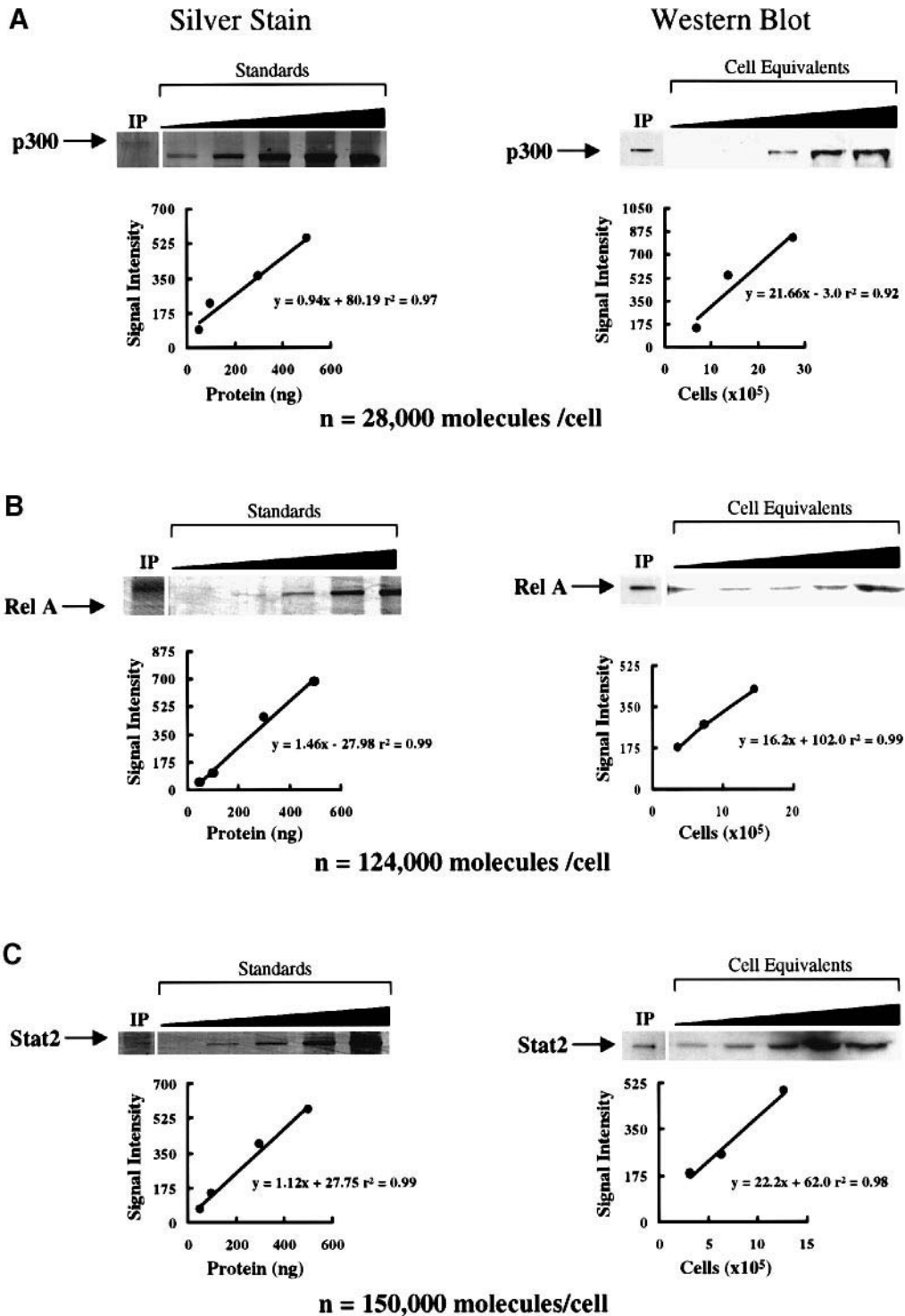
TNF-α has been shown to exert a cytotoxic effect on

Fig. 8. Quantitative analysis of nuclear p300, RelA and Stat2. Determination of the molar ratios between p300, RelA and Stat2 in the nucleus. Jurkat cells (8.7×10^7) were left unstimulated or stimulated with 1000 U/ml IFN-α for 18 h or 100 U/ml TNF-α for 4 h. The number of molecules in the immunoprecipitations was determined by comparing the signal intensity of immunoprecipitated protein from 200 μg of nuclear extract with standards on a silver-stained SDS–PAGE. For p300, the comparison was made with 50, 100, 300, 500 and 750 ng of myosin H-chain (**A**); for RelA after stimulation with TNF-α, with 10, 50, 100, 300 and 500 ng of phosphorylase B (**B**); and for Stat2 after stimulation with IFN-α, with 10, 50, 100, 300 and 500 ng of phosphorylase B (**C**). IP indicates the amount of proteins obtained by using 200 μg of nuclear extract for the immunoprecipitations. Different dilutions of IP were analyzed for a better analysis (data not shown). The slope of silver-stained RelA protein was 1.51 in comparison with 1.46 for phosphorylase B, 1.09 for Stat2 in comparison with 1.12 for phosphorylase B and 0.81 for p300 in comparison with 0.94 for myosin H-chain. Equal amounts of immunoprecipitated proteins were then compared in a Western blot analysis with 2, 5, 10, 20 and 40 μg of nuclear extract from a known number of cells. Numbers of molecules per cell were obtained by dividing the numbers of molecules in the immunoprecipitates by the number of cells producing the same signal intensity.

tumor cells (Carswell *et al*., 1975; Havell *et al*., 1988). In several cell types, where TNF-α alone cannot induce apoptosis, the apoptotic response can be activated when NF-κB function is absent or inhibited (Beg and Baltimore, 1996; van Antwerp *et al*., 1996; Wang *et al*., 1996). The finding that IFN-α may inhibit specifically the action of NF-κB in TNF-α-stimulated cells raises the possibility that it may block the anti-apoptotic effects of NF-κB in malignant cells and improve the efficacy of its anti-tumor effect. In complex cellular promoters where other important cytokine-responsive elements may direct a more intricate pattern of regulation, the combined effects of IFN-α and TNF-α may not always lead to transcriptional inhibition because these cytokines subsequently could induce other transcription factors which would result in gene activation. Synergy might thus be expected on some promoters, while competitive interaction would be expected for those that are primarily κB- or Stat2-dependent as shown here for the HIV LTR.

A number of studies have investigated the effect of IFN-α on HIV-1 replication. This inhibitory effect has been demonstrated in peripheral blood mononuclear cells, T lymphoblast and monocytoid cell lines (Hartshorn *et al*., 1987; Gendelman *et al*., 1990a; Kornbluth *et al*., 1990; Brinchmann *et al*., 1991; Shirazi and Pitha, 1992). IFN-α has been shown to affect different steps of the HIV-1 replication cycle, including events prior to integration (Gendelman *et al*., 1990b; Meylan *et al*., 1993; Baca-Regen *et al*., 1994), and transcriptional or post-transcriptional controls (Poli *et al*., 1989; Smith *et al*., 1991; Popik and Pitha, 1992; Coccia *et al*., 1994; Agy *et al*., 1995; for review see Pitha, 1991). Transcriptional inhibition of the HIV replication life cycle by competitive binding of transcription factors provides an additional mechanism to explain the inhibition of HIV-1 gene expression by IFN-α. Negative regulation of NF-κB by IFN-α through its coactivators could explain its ability to inhibit HIV transcription in Jurkat T-leukemia cells and hence may partly contribute to its decrease in replication. Our observation is in agreement with previous findings that IFN-α can inhibit HIV transcriptional activation by phorbol esters in a cell line containing a Tat-defective provirus. Co-transfection of a Tat expression plasmid into this cell line not only activated transcription of the provirus but also overcame the inhibitory effect of IFN-α on transcription (Popik and Pitha, 1992). Taken together, these results suggest that Tat may help to overcome transcriptional inhibition by IFN-α.

In many signal transduction pathways, interactions between signaling protein kinases and phosphatases modulate effects induced by divergent cytokine receptors. In the case of IFN-α and JAK/STAT signaling, the Stat transcription factor transduces information from a specific receptor into the nucleus (Darnell *et al*., 1994; Shuai *et al*., 1994; Schindler and Darnell, 1995; Qureshi *et al*., 1996) of Stat by kinases from other signaling pathways in the cytoplasm less likely to occur. Thus, cells may have evolved other mechanisms to coordinate the response to divergent signals in the nucleus. Although other transcription factors such as CREB, c-Jun, c-Fos, YY1 and c-Myb have been shown to bind to p300/CBP, these factors respond to distinct signal transduction pathways and bind to sites distinct from Stat2 and RelA (Bhattacharya *et al*.,

1996; Eckner, 1996; Perkins *et al*., 1997), suggesting that their interactions with different regions of p300/CBP are likely to control their distinct target gene products. At the same time, it is also likely that these other transcription factors may show specific competition among themselves for binding to different sites on p300/CBP through an analogous mechanism. Thus, p300/CBP could serve to integrate information from distinct signaling pathways from different cytokines in order to coordinate the transcriptional regulation of relevant genes. Whether p300 and CBP or other family members such as p400 (Howe and Bayley, 1992; Lill *et al*., 1997b) can compensate for each other can be addressed with genetically deficient cell lines or mice, which are not yet available. This is likely to be the case in Rubenstein–Taybi syndrome (Petrij *et al*., 1995), where haplo-insufficiency of CBP is the apparent cause of developmental defects, supporting the idea that coactivator levels may be rate limiting for cellular gene expression. Our data suggest, in addition, that the amount of p300, CBP or p400 is insufficient to compensate functionally for the limiting amount of coactivator relative to the two transcription factors RelA and Stat2. The interaction between these transcription factors and their coactivators may also play a role in the regulation of other viral and cellular genes which are similarly controlled by NF-κB.

Materials and methods

Plasmids

HIV–CAT, ΔκB HIV–CAT and RSV–CAT have all been described previously (Gorman *et al*., 1982; Nabel and Baltimore, 1987; Leung and Nabel, 1988). OAS–CAT contains the CAT gene regulated by the 2′–5′ oligoadenylate synthetase promoter, which was generated by PCR using primers GGAATTTCCATATGGATCAGGGGAGTGTCT and CCGGAATTCATTGACAGGAGAGAGAG and genomic DNA from Jurkat cells. The PCR products were digested with *Nde*I and *Eco*RI and ligated into RSV–CAT, which had been cut with the same enzymes. α-IRE–CAT was generated by ligating the double-stranded oligonucleotide TATGAGGAAACGAAACCAGATATCTGAGGAAA-CGAAACCAG into RSV–CAT that had been cut with *Nde*I and *Eco*RI, which removed the RSV enhancer element. GAS/α-IRE–CAT was generated by ligating the double-stranded oligonucleotide TATGAAGTACTTTCAGTTTCATATTACTCTAAGTACTTTCAGTTT-CATATTACTCTAGAT into α-IRE–CAT that had been cut with *Nde*I and *Eco*RV. RSV–RelA has been described previously (Perkins *et al*., 1994a).

The cDNA insert for pGex–p300 (CH1) was generated by PCR using primers ATGGGATCCATGGGTCAACAGCCAGCCCCG and AGA-GAATTCTCAATCACCAGCATTTTTGAGGGGGAG and the p300-encoding template described in Perkins *et al*. (1997). The PCR products were digested with *Bam*HI and *Eco*RI and ligated into pGEX–CD1, which had been cut with the same enzymes. pGEX–p300 (CH1) contains amino acid residues 302–422. Bluescript RelA used for *in vitro* transcription/translation has been reported previously (Perkins *et al*., 1994b). The cDNA of the Stat2 transactivation domain (amino acids 670–851) was obtained by RT–PCR from 0.5 μg of total RNA from Jurkat cells (Trizol, Gibco-BRL) using oligo(dT) as a primer and reverse transcriptase (Perking Elmer) using primers GCTAGGTCGAC-CACCATGGATGAAGCTTTTGGGTGCTAC and CCGGAATTCCTA-GAAGTCAGAAGGCATCAAG. The RT–PCR products were digested with *Sal*I and *Eco*RI and ligated into pBS, which had been cut with the same enzymes downstream of the T7 promoter.

GST–Stat2(TA) was generated by digesting the RT–PCR products with *Sal*I and *Eco*RI and ligated into pGEX–CD1, which had been cut with *Xho*I and *Eco*RI. pGEX–Stat2 contains amino acid residues 670–851 of Stat2. GST–Stat2(TA)ΔC was generated by digesting GST–Stat2(TA) with *Nsi*I and *Eco*RI. Ends subsequently were blunt ended with T4 polymerase and religated. pGEX–Stat2ΔC contains amino acid residues 670–768 of Stat2. pGAL4–Stat2(TA) was constructed by cloning

a *Hinc*II–*Xba*I fragment from the above described pBS–Stat2 into pRSVGAL4.1 cleaved with *Sma*I and *Xba*I. pGAL4–Stat2(TA) contains amino acid residues 670–851 of Stat2. pRSVGAL4.1 was constructed by ligating the promoter of pRc/RSV (Invitrogen) as an *Ssp*I–*Hin*dIII fragment into pSG424 that had been cut with *Ssp*I–*Hin*dIII. pGAL4–Stat2(TA)ΔC was generated by digesting pGAL4–Stat2(TA) with *Xcm*I and *Bam*HI. Ends subsequently were blunt ended with T4 polymerase and religated. pGAL4–Stat2(TA)ΔC contains amino acid residues 670–768 of Stat2. pCMV–p300 and pCMV–AP plasmids, together with the GAL–CAT reporter plasmid (pG5E–CAT) and the pSG424 GAL4 expression plasmid, have all been described previously (Lillie and Green, 1989; Sadowski and Ptashne, 1989; Perkins *et al.*, 1997). The deletion mutant p300 (amino acids 1–596) and the deletion mutant p300 (amino acids 1–349) were generated by digesting pCMV-p300 with *Bam*HI and *Xba*I or *Sph*I and *Xba*I. Ends subsequently were blunt ended with T4 polymerase and religated.

Transfections and reporter assays

Jurkat T-leukemia cells were transfected using DEAE-dextran, cells were harvested after 24 h, and CAT assays were performed as previously described (Leung and Nabel, 1988). RelA transfection of 293 cells was performed as described previously (Perkins *et al.*, 1997). Cells were stimulated with either TNF-α (Genzyme) or IFN-α (Referone-A, Roche) as indicated. Luciferase activity was measured according to Aguilar-Cordova *et al.* (1994).

Electrophoretic mobility shift assays

Nuclear extracts were prepared and analyzed by EMSA for κB binding activity as described previously (Shu *et al.*, 1993; Perkins *et al.*, 1994a). Cells were harvested and nuclear extracts (5 μg) were analyzed using a ^{32}P-labeled double-stranded κB oligonucleotide probe. Unlabeled wild-type and mutant competitor DNAs (20 ng) were included as indicated.

Protein binding assays and Western blots

RelA immunoprecipitations of 293 cells were performed as described previously (Perkins *et al.*, 1997) with 1 μg of an anti-RelA antibody (Santa Cruz #sc109) or control rabbit IgG. Deletion analysis of the N-terminal fragments of p300 was performed by digesting pBS–p300 (Perkins *et al.*, 1997) with *Sca*I, *Nde*I or *Sph*I, prior to *in vitro* transcription/translation (radiolabeled T7 TNT reticulocyte transcription/translation system, Promega) to produce p300 fragments truncated at amino acids 1031, 610 or 347, respectively. RelA and Stat2 immunoprecipitations of Jurkat cells were performed with 1.5 μg of an anti-RelA antibody (Santa Cruz #sc109), an anti-Stat2 antibody (Santa Cruz #sc476) or control rabbit IgG. GST–CH1 was expressed and purified as reported (Smith and Johnson, 1988). Beads containing fusion proteins subsequently were incubated with the indicated radiolabeled TNT rabbit reticulocyte proteins. Purified GST was used as the control. p300 immunoprecipitations were performed as described previously (Perkins *et al.*, 1997) with 1 μg of an anti-p300 antibody (PharMingen #14991A) or control rabbit IgG in the presence of 250 mM NaCl to eliminate binding of associated endogenous factors (such as RelA, Stat2, Cdk2, cyclin E). Beads containing p300 subsequently were pre-incubated with GST–Stat2(TA) (1 μg) or GST–Stat2(TA)ΔC (1 μg) for 30 min at 4°C, in the presence of 75 mM NaCl, followed by an incubation with radiolabeled *in vitro* translated RelA for 2 h at 4°C. The assay was terminated by the addition of SDS gel loading buffer to the samples. Samples were resolved on a 10% SDS–polyacrylamide gel, which was then dried and subjected to autoradiography. Western blot analysis was performed as described (Perkins *et al.*, 1997) using anti-p300 antibody (Santa Cruz #sc584 and #sc585), anti-RelA antibody (Santa Cruz #sc109) and anti-Stat2 antibody (Santa Cruz #sc476 and #sc839).

Acknowledgements

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