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Coordination of a Transcriptional Switch by HMGI(Y) Acetylation

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Dynamic control of interferon-β (IFN-β) gene expression requires the regulated assembly and disassembly of the enhanceosome, a higher-order nucleoprotein complex formed in response to virus infection. The enhanceosome activates transcription by recruiting the histone acetyltransferase proteins CREB binding protein (CBP) and p300/CBP-associated factors (PCAF)/GCN5, which, in addition to modifying histones, acetylate HMGI(Y), the architectural component required for enhanceosome assembly. We show that the accurate execution of the IFN-β transcriptional switch depends on the ordered acetylation of the high-mobility group I protein HMGI(Y) by PCAF/GCN5 and CBP, which acetylate HMGI(Y) at distinct lysine residues on endogenous promoters. Whereas acetylation of HMGI(Y) by CBP at lysine-65 destabilizes the enhanceosome, acetylation of HMGI(Y) by PCAF/GCN5 at lysine-71 potentiates transcription by stabilizing the enhanceosome and preventing acetylation by CBP.

Gene-specific transcriptional switches are thought to be generated through the dynamic assembly and disassembly of transcription factor-enhancer DNA complexes, although the mechanisms controlling these processes in real time are poorly understood. Virus-induced activation of the IFN-β gene represents one of the best characterized transcriptional switches in eukaryotic cells (*1*). A 65-bp enhancer element contains the necessary information for directing the assembly of a virus-induced enhanceosome consisting of NF-κB, IRFs, ATF-2/cJun, and the architectural protein HMGI(Y), which orchestrates this process by mediating a network of protein-DNA and protein-protein interactions (*2, 3*). The enhanceosome is assembled in the nucleosome-free enhancer region, and it activates transcription by instructing a recruitment program of chromatin-modifying activities that target a strategically positioned nucleosome masking the TATA box and start site of transcription (*4*). The first step involves recruitment of the GCN5/PCAF complex, which acetylates the nucleosome, and this is followed by recruitment of the CBP-PolII holoenzyme complex (*4–7*). Nucleosome acetylation, in turn, facilitates SWI/SNF recruitment by CBP, resulting in chromatin remodeling and binding of TFIID to the promoter (*4*).

Consistent with the observation that histone acetylation takes place at the IFN-β promoter is the fact that the histone acetyltransferase (HAT) activities of both CBP and GCN5/PCAF are required to attain maximum levels of virus-induced IFN-β transcription (*8, 9*). However, CBP and GCN5/PCAF also acetylate HMGI(Y) at distinct lysine residues (K65 and K71, respectively), causing distinct effects on transcription. Acetylation of HMGI(Y) by CBP decreases its affinity for DNA, resulting in enhanceosome destabilization and transcriptional shutoff (*8*). Here, we show that acetylation of HMGI(Y) by GCN5/PCAF strengthens enhanceosome assembly and protects the enhanceosome from premature disruption by CBP-dependent acetylation of HMGI(Y).

To examine the role of K71 acetylation, an HMGI(Y) derivative in which K71 was mutated to arginine was used in cotransfection experiments along with an IFN-β reporter plasmid with or without a PCAF or PCAF HAT$^-$ expression vector (*10*). These experiments revealed that expression of the mutant HMGI(Y) with or without the PCAF expression vectors strongly reduced the levels of virus-induced transcription throughout the time course, especially at early time points (e.g., 9 hours), and thus suggested a link between PCAF HAT activity and HMGI(Y) acetylation at K71, in addition to the link between PCAF HAT activity, histone acetylation, and IFN-β gene expression (*4, 10*). Therefore, because acetylation of HMGI(Y) by PCAF does not alter the affinity of HMGI(Y) for DNA (*8*) and because K71 lies in one of the critical protein-protein interaction domains of HMGI(Y) (*3*), we tested the effect of PCAF acetylation on the ability of HMGI(Y) to interact with the IFN-β activators. Recombinant wild-type (WT) or mutant HMGI(Y) proteins were radiolabeled by acetylation using PCAF or CBP in the presence of ^{3}H-labeled acetyl-CoA, and the resulting mixture of ^{3}H-labeled (~10 to 20%) and nonlabeled HMGI(Y) proteins was tested for interaction with the activators by glutathione *S*-transferase (GST) interaction experiments (*11*). Fluorography revealed that the fraction of HMGI(Y) protein radiolabeled by PCAF acetylation, but not by CBP acetylation, preferentially interacted with all activators (Fig. 1A, compare lanes 1 through 5 with 6 through 10). Parallel Western blot analysis revealed that CBP-acetylated HMGI(Y) interacted with the activators as efficiently as the unacetylated protein (Fig. 1A, lanes 22 through 33), thus ruling out the possibility that HMGI(Y) acetylation by CBP decreases its affinity for the activators. In addition, PCAF-acetylated and ^{3}H-labeled HMGI(Y)(K71R) did not preferentially interact with the activators (Fig. 1A, lanes 11 through 15), although PCAF-acetylated ^{3}H-labeled HMGI(Y) (K65R) interacted with the activators as efficiently as PCAF-acetylated wild-type HMGI(Y) (Fig. 1A, compare lanes 1 through 5 with 16 through 21).

To further support the conclusions from these experiments, three peptides were synthesized that encompassed the protein-protein interaction domain of HMGI(Y) but differed only in their acetylation state (Fig. 1B). These peptides were tested for their ability to compete with in vitro translated ^{35}S-labeled HMGI(Y) for interaction with p50 (*11*). Whereas neither the unacetylated nor the K65-acetylated peptide could significantly compete with HMGI(Y) for interaction with p50 at these concentrations (Fig. 1C, lanes 1

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through 8), the K71-acetylated peptide disrupted the interactions efficiently and in a dose-dependent manner (lanes 9 through 11). We suggest that K71 acetylation either neutralizes a positive charge that could decrease protein-protein interactions or creates a novel interaction interface on HMGI(Y) for the activators. Taken together, these experiments suggest that HMGI(Y) acetylation by PCAF may facilitate enhanceosome assembly. DNase I footprinting experiments showed that acetylation of HMGI(Y) by PCAF increased its ability to promote enhanceosome assembly as compared to unacetylated HMGI(Y) (Fig. 1D, compare lanes 12 through 14 with 9 through 11) (*12*). This effect is due to specific acetylation at K71, because the ability of the HMGI(Y)(K71R) protein to promote enhanceosome assembly was not enhanced by PCAF acetylation (*10*). Thus, acetylation of HMGI(Y) by PCAF at K71 facilitates enhanceosome assembly by increasing HMGI(Y)'s affinity for the activators.

To investigate real-time enhanceosome assembly in vivo, we carried out chromatin immunoprecipitation (IP) experiments (*13*). Figure 2A shows that each of the IFN-β activators is recruited to the enhancer with distinct kinetics. For example, p65 is initially detected at the IFN-β promoter at 2 hours after infection together with IRF-1, whereas ATF-2 is recruited to the promoter an hour later followed by the arrival of IRF-3 and c-Jun to the enhanceosome after another hour (Fig. 2A). IRF-7 is the last transcription factor that binds to the IFN-β enhancer, a result consistent with the fact that IRF-7 is synthesized in response to virus infection via the IFN autocrine loop (*14*). Thus, these experiments revealed that the enhanceosome is sequentially constructed via stepwise cooperative interactions, indicating an unexpected degree of plasticity in its assembly. The earliest time point at which the full IFN-β enhanceosome assembles is at 4 to 5 hours after infection, which is the time point at which the GCN5/PCAF complex is recruited to the enhanceosome (*4*). Furthermore, our experiments show that the enhanceosome forms transiently, because it begins to disassemble at 12 hours after infection, con-

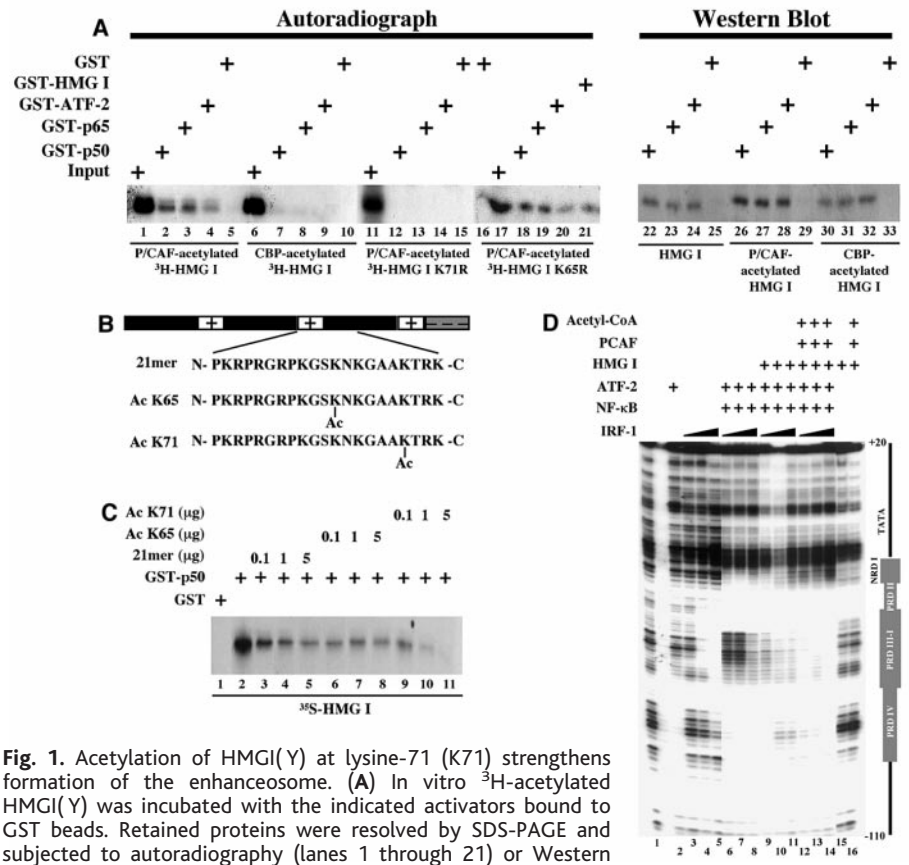

Fig. 1. Acetylation of HMGI(Y) at lysine-71 (K71) strengthens formation of the enhanceosome. (**A**) In vitro ^{3}H-acetylated HMGI(Y) was incubated with the indicated activators bound to GST beads. Retained proteins were resolved by SDS-PAGE and subjected to autoradiography (lanes 1 through 21) or Western blot analysis with αHMGI(Y) antibody (lanes 22 through 33). (**B**) Diagram illustrating the three peptides synthesized for these experiments (*16*). (**C**) In vitro translated ^{35}S-HMGI(Y) was incubated with GST-p50 and the indicated amounts of 21mer, AcK65, or AcK71 peptides. (**D**) DNase I footprinting experiments using PCAF- or mock-acetylated HMGI(Y) (100 ng) and recombinant IFN-β activators (30 ng NF-κB, 400 ng ATF-2/c-JUN, and 30, 100, or 200 ng IRF-1). Quantitation revealed that unacetylated HMGI(Y) promoted enhanceosome assembly fourfold, whereas PCAF-acetylated HMGI(Y) promoted assembly 20-fold.

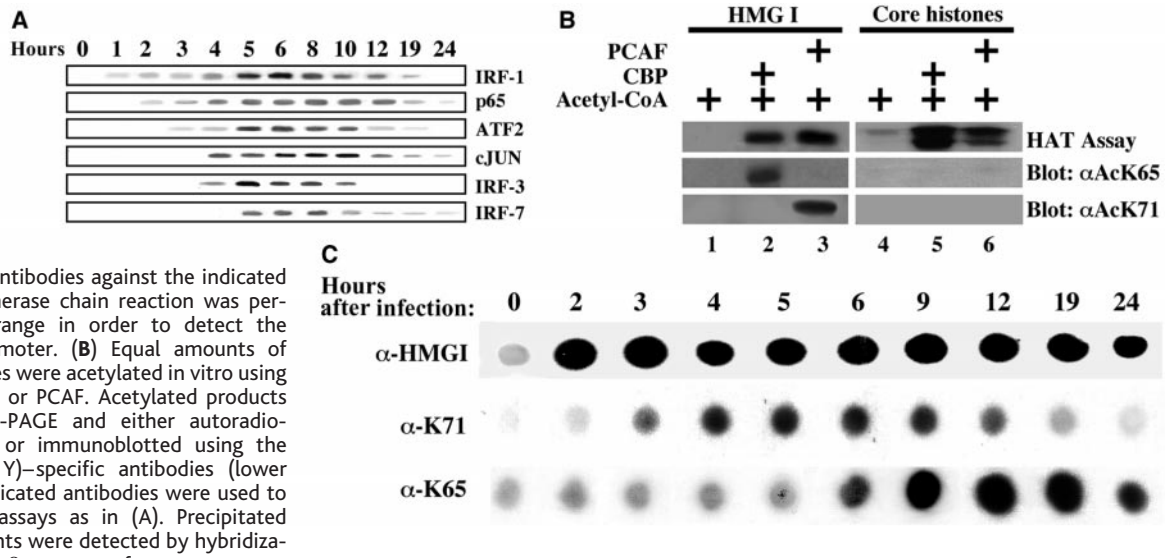

Fig. 2. HMGI(Y) undergoes an ordered cascade of virus-induced acetylation at the IFN-β promoter in vivo. (**A**) HeLa cells were infected with Sendai virus for the indicated amounts of time and the chromatin was crosslinked and precipitated using antibodies against the indicated IFN-β activators. Polymerase chain reaction was performed in the linear range in order to detect the precipitated IFN-β promoter. (**B**) Equal amounts of HMGI(Y) or core histones were acetylated in vitro using either recombinant CBP or PCAF. Acetylated products were resolved by SDS-PAGE and either autoradiographed (upper panel) or immunoblotted using the indicated acetyl-HMGI(Y)–specific antibodies (lower two panels). (**C**) The indicated antibodies were used to perform chromatin IP assays as in (A). Precipitated IFN-β promoter fragments were detected by hybridization using a labeled IFN-β promoter fragment.

sistent with our previous predictions (*8*).

To test whether HMGI(Y) acetylation by GCN5/PCAF correlates with enhanceosome assembly, we carried out chromatin IP experiments using antibodies distinguishing between K71- and K65-acetylated HMGI(Y) (*13, 15*). The antibody raised against the K65-acetylated HMGI(Y) peptide specifically recognized CBP-acetylated HMGI(Y) (Fig. 2B, lane 2) and not PCAF-acetylated HMGI(Y) (lane 3). Similarly, PCAF-acetylated HMGI(Y) was recognized only by the antibody raised against the K71-acetylated HMGI(Y) peptide (lane 3). None of the antibodies cross-reacted with CBP- or PCAF-acetylated core histones (lanes 4 through 6). The chromatin IP experiment of Fig. 2C, using the generic HMGI(Y) antibody, revealed that the protein associates with the enhancer at 2 hours after infection, roughly at the time of association of p65, ATF-2, and IRF-1 with the promoter (Fig. 2A). This observation is consistent with previous in vitro data showing that nonacetylated HMGI(Y) can recruit these factors to the promoter (*3*). Figure 2C also shows that HMGI(Y) acetylation at K71 is first seen at 3 hours after infection, peaks at 4 to 6 hours, and then disappears. Thus, K71 HMGI(Y) acetylation perfectly correlates with the onset of enhanceosome assembly in vivo (Fig. 2, A and C), and it occurs at the exact time at which GCN5 is recruited to the IFN-β promoter (*4*). By contrast, acetylation of HMGI(Y) at K65 is not detected sooner than 6 hours after infection, despite the fact that CBP is already at the promoter (*4*). K65 acetylation peaks between 12 and 19 hours after infection, which coincides with the onset of enhanceosome disruption (Fig. 2A), thus verifying previous in vitro observations (*8*).

Next, we examined the relationship between acetylation of HMGI(Y) at K71 and K65. We used the three peptides shown in Fig. 1B and recombinant CBP or P/CAF HAT domain proteins in HAT assays (*10*). We found that the K71-acetylated peptide was acetylated at K65 by CBP HAT fivefold less efficiently when compared to the unacetylated peptide, whereas when P/CAF HAT was used to acetylate the WT 21-residue oligomer (21mer) and Ac K65 peptides, no significant difference was noted (*10*). Thus, acetylation of HMGI(Y) at K71 lowers the efficiency with which CBP can acetylate HMGI(Y) at K65, although acetylation of HMGI(Y) at K65 does not affect PCAF's ability to acetylate HMGI(Y) at K71.

Because CBP acetylation of HMGI(Y) at K65 destabilizes the enhanceosome (*8*), we reasoned that K71-acetylated HMGI(Y) might interfere with enhanceosome disruption. To test this hypothesis, we carried out DNase I footprinting experiments using the three peptides shown in Fig. 1B to direct enhanceosome assembly (*12*). Enhanceosomes formed with each of the three peptides were challenged with recombinant CBP in the presence or absence of acetyl-CoA. The unacetylated WT 21mer peptide directed enhanceosome formation (Fig. 3, lanes 9 through 15), and these enhanceosomes were disrupted upon addition of CBP and acetyl-CoA (lanes 16 through 22). In contrast, when CBP and acetyl-CoA were added to enhanceosomes assembled with the Ac K71 peptide, virtually no disruption was observed (compare lanes 23 through 28 with 29 through 35). Underscoring the specificity of this phenomenon, no destabilization was seen upon the addition of CBP and acetyl-CoA to enhanceosomes formed with the Ac K65 peptide (compare lanes 36 through 42 with 43 through 49), although these enhanceosomes were highly unstable to begin with (compare lanes 9 through 15 with 36 through 42). Taken together, these data demonstrate that the early acetylation of HMGI(Y) at K71 by GCN5/PCAF not only facilitates enhanceosome assembly, it also protects the enhanceosome from disruption by inhibiting CBP-induced acetylation at K65.

Our findings demonstrating regulated acetylation of HMGI(Y) by CBP and GCN5/PCAF within the enhanceosome disclose an unprecedented example of the flexibility required for precise control of gene transcription. The early "core" enhanceosome bearing HMGI(Y), ATF-2, NF-κB, and IRF-1 is "enriched" sequentially with IRF-3, c-Jun, and IRF-7 in a way that ensures maintenance of the higher-order three-dimensional structure, despite continuous changes in shape that correlate with the ordered recruitment of distinct coactivator complexes (*4*). We propose here that the accurate execution of the IFN-β tran-

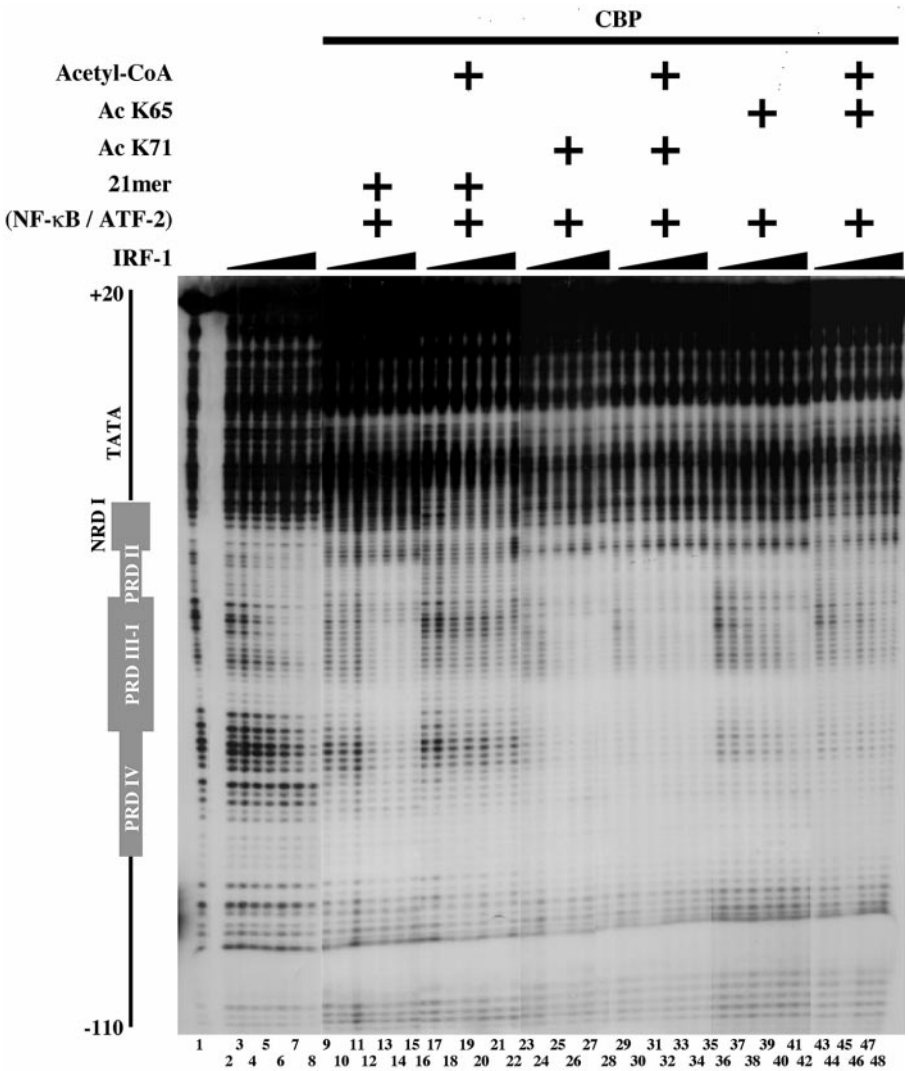

Fig. 3. CBP does not disrupt enhanceosomes that contain PCAF-acetylated HMGI(Y). DNase I footprinting assays were carried out essentially as in Fig. 1D, using the peptides (100 ng) acetylated in situ instead of preacetylated full-length HMGI(Y).

scriptional switch depends on the ordered recruitment of GCN5/PCAF and CBP, which acetylate HMGI(Y) at distinct lysine residues, inducing opposite effects on enhanceosome stability. It takes approximately 4 hours after viral infection to assemble the enhanceosome and 2 more hours to synthesize the first IFN-β transcripts. We speculate that each of the transcription factors associates with the enhancer only weakly at the onset of viral infection, despite the presence of HMGI(Y), because it is not acetylated by GCN5/PCAF. Then, GCN5/PCAF is recruited and acetylates histones and HMGI(Y) at K71, thus "locking" the enhanceosome into a "metastable" configuration that initiates subsequent steps of the recruitment program, such as CBP-PolII, SWI/SNF, and TFIID recruitment (*4*). Thus, at the time of peak HMGI(Y) K71 acetylation (5 hours after infection), all IFN-β activators are found on the enhancer at their highest amounts, indicating stable enhanceosome assembly. However, CBP recruited to the enhanceosome cannot acetylate HMGI(Y) at K65, because HMGI(Y) is already acetylated at K71 by GCN5/PCAF. K65 acetylation and subsequent enhanceosome disassembly correlate with K71 deacetylation. Thus, the ordered and highly controlled acetylation of HMGI(Y) by two distinct HAT coactivators coordinates the IFN-β transcriptional switch by instructing either enhanceosome assembly or disassembly.

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10. Relevant data and experimental procedures can be found at *Science* Online at www.sciencemag.org/cgi/content/full/293/5532/1133/DC1.
11. HMGI(Y) derivatives were acetylated in vitro as previously described (*8*) with the following modifications. Substrates were incubated with enzyme and ^{3}H-labeled acetyl CoA (Sigma, St. Louis, MO) for 1 hour, and fresh enzyme was added every hour for 4 hours. Using ^{3}H-labeled HMGI(Y) proteins, GST pull-down assays were performed essentially as described (*3*). For peptide competition assays, the indicated amount of peptide (obtained from Research Genetics, Huntsville, AL) was added during incubation of GST-p50 with in vitro translated ^{35}S-labeled HMGI(Y) (TnT in vitro translation kit; Promega, Madison, WI).
12. DNase I footprinting experiments were carried out as described (*2*). For reactions containing preacetylated HMGI(Y), acetylation was carried out as described above (*11*) except that cold acetyl-CoA (Pharmacia, Peapack, NJ) was used in these reactions. HMGI(Y) was acetylated in situ (Fig. 3) according to methods in (*8*).
13. Chromatin IP experiments were carried out as described (*4*).
14. M. Sato *et al.*, *Immunity* **13**, 539 (2000).
15. Acetylated peptides were synthesized for K65 (Genosys, The Woodlands, TX) and K71 (Research Genetics) in order to immunize rabbits. Whole rabbit serum was purified by passing it over a column conjugated to the acetylated peptide and then passing the eluant over a column conjugated to the unacetylated peptide in order to deplete nonspecific antibodies. The flow-through was collected and used in Western blots and chromatin IP experiments (Fig. 2, B and C).
16. Single-letter abbreviations for the amino acid residues are as follows: 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.

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Cytokine-Specific Transcriptional Regulation Through an IL-5Rα Interacting Protein

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Cytokine receptors consist of multiple subunits, which are often shared between different receptors, resulting in the functional redundancy sometimes observed between cytokines. The interleukin 5 (IL-5) receptor consists of an IL-5–specific α-subunit (IL-5Rα) and a signal-transducing β-subunit (βc) shared with the IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) receptors. In this study, we sought to find a role for the cytoplasmic domain of IL-5Rα. We show that syntenin, a protein containing PSD-95/Discs large/zO-1 (PDZ) domains, associates with the cytoplasmic tail of the IL-5Rα. Syntenin was found to directly associate with the transcription factor Sox4. Association of syntenin with IL-5Rα was required for IL-5–mediated activation of Sox4. These studies identify a mechanism of transcriptional activation by cytokine-specific receptor subunits.

The IL-5Rα is expressed on B cells and on eosinophilic and basophilic granulocytes (*1*–*3*), and IL-5 mediates the differentiation and survival of eosinophils (*4*). Furthermore, IL-5 induces the proliferation of progenitors of B1 cells and induces differentiation of mature B1 cells and conventional B2 cells into immunoglobulin-producing cells (*5*, *6*). We reasoned that if the specific α chains of cytokine receptors play a role in signaling, this may be mediated by α chain–associated proteins. However, the identity of such proteins has remained elusive. To identify proteins specifically mediating IL-5Rα signaling, we performed a two-hybrid screen using the IL-5Rα cytoplasmic domain. We constructed a two-hybrid cDNA library from human granulocytes known to express the IL-5Rα (*7*). Syntenin, protein containing tandem PDZ domains, associated specifically with the cytoplasmic domain of IL-5Rα in yeast (Fig. 1A). In glutathione *S*-transferase (GST) co-precipitation (pull-down) analyses (*8*), IL-5Rα associated with syntenin in vitro (Fig. 1B). We tested whether the association between syntenin and IL-5Rα was direct with the use of BIAcore surface plasmon resonance technology. Syntenin associated with immobilized GST–IL-5Rα$_{cyt}$ fusion protein with a calculated dissociation constant (K_d) value of 470 nM (Fig. 1C, upper panel). No binding was observed between syntenin and immobilized GST (Fig. 1C, lower panel). Syntenin, therefore, associates directly with IL-5Rα$_{cyt}$, and the binding affinity observed is similar to that obtained for the interaction of isolated PDZ domains with optimized peptides containing consensus PDZ-binding motifs (*9*). Syntenin co-immunoprecipitated with IL-5Rα from mammalian cells, indicating that the protein association can occur in vivo (Fig. 1D). Syntenin did not bind to GST fusion proteins derived from either the IL-3 or the GM-CSF receptor (*10*).

Deletion mutants of IL-5Rα were then used to identify the region of interaction with syntenin. Deletion of the last 15 carboxyl-terminal residues abolished the association with syntenin (Fig. 2A). A comparison of the amino acid sequences between mouse and human IL-5Rα revealed that although these sequences were somewhat variant, two of the four COOH-ter-

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