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Tip60 Interacts with Human Interleukin-9 Receptor α-Chain

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Interleukin-9 (IL-9) exerts its pleiotropic effects through the IL-9 receptor (IL-9R) complex that consists of the ligand specific IL-9R α-chain, and the IL-2R γ-chain. In this study, we used a modified yeast two-hybrid system to isolate cDNAs encoding proteins that interact with the intracellular domain of the human IL-9R α-chain (hIL-9Rα). We have identified Tip60, an HIV-1 Tat transcription cofactor, as an hIL-9Rα interacting protein. The interaction between hIL-9Rα and Tip60 was confirmed by coimmunoprecipitation and colocalization studies. This is the first demonstration that Tip60 associates with a membrane receptor. We also mapped amino acids 411–423 in hIL-9Rα and amino acids 100–147 in Tip60 to be important for interaction. Interestingly, the region in hIL-9α that binds Tip60 is adjacent to the site previously shown to interact with Stat3. Tip60 binds HIV-Tat and mediates Tat-dependent transactivation possibly through its histone acetyltransferase activity. Our results therefore suggest that Tip60 may act as a cofactor of Stat3 or as an adaptor protein for molecules that are important for IL-9 signaling.

Interleukin-9 (IL-9) is a multifunctional T cell derived cytokine with a variety of biological activities in different cell lines and tissues (for review see 1, 2). IL-9 exerts its biological effects by binding to the IL-9 receptor (IL-9R) that belongs to the IL-2 receptor γ-chain superfamily including receptors for IL-2, IL-4, IL-7, IL-9 and IL-15 (3). All these receptors have a common IL-2R γ-chain and at least one ligand-specific subunit (3). These receptors also share certain signal transduction pathways, such as activation of JAK/STAT pathway, phosphorylation of IRS-1 and IRS-2, and expression of nuclear proto-oncogenes (4–8). In addition, specific cytoplasmic domains of human IL-9Rα chain (hIL-9Rα) important for cell proliferation, anti-apoptotic activity, growth regulation, STAT and IRS phosphorylation have been determined (6, 7).

Tip60 was originally isolated as an HIV-1 Tat interactive protein and has been shown to increase Tat-dependent transcriptional activation (9). Tip60 and its homologues, such as yeast Sas2, Sas3 (10), ESA1 (11), human MOZ (12), and Drosophila MOF (13), contain a conserved domain identified in various histone acetyltransferases (14). Many studies have demonstrated that histone acetyltransferase activity of various transcriptional factors, such as Ccnp5, CBP, P/CAF and TAF_{250}, may play an important role in gene activation (for review see 15–17). Tip60 is expressed in a wide variety of cell types and has been shown to have histone acetyltransferase activity in vitro (18).

To identify proteins involved in IL-9R signaling, we performed yeast two-hybrid screen to identify molecules that can bind to the intracellular domain of hIL-9Rα. One of the positive clones we isolated encoded Tip60 protein (9). In this study, we confirmed the interaction between hIL-9Rα and Tip60 in cells by coimmunoprecipitation and colocalization studies. Based on the regions important for the interaction, we suggest that Tip60 may play a dual role in IL-9 mediated biological functions.

MATERIALS AND METHODS

Reagents. Monoclonal anti-phosphotyrosine and anti-c-Myc (9E10) antibodies were from Upstate Biotechnology, Inc. (Lake Placid, NY), and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively. Monoclonal anti-hIL-9Rα and anti-Flag M2 antibodies were purchased from R & D Systems (Minneapolis, MN) and Eastman Kodak

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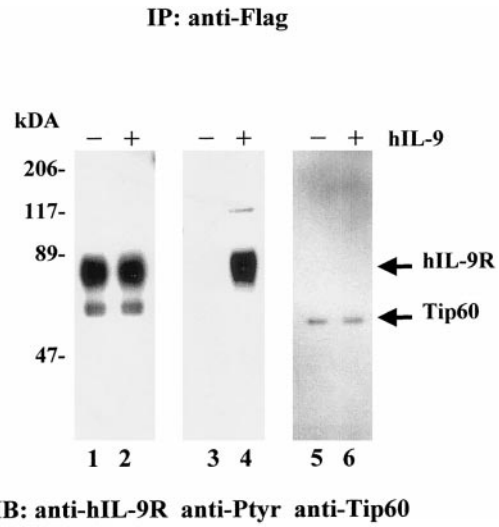


FIG. 1. Tyrosine phosphorylation is not required for the interaction between Tip60 and hIL-9Rα. TS-1 cells expressing wild type Flag-tagged hIL-9Rα were treated for 5 minutes with hIL-9 (100 ng/ml), and total cell lysates were immunoprecipitated with anti-Flag antibodies. Immunoprecipitates were subjected to SDS-PAGE and immunobloted with anti-hIL-9R (lanes 1, 2), anti-phosphotyrosine (lanes 3, 4), and anti-Tip60 (lanes 5, 6) antibodies, respectively.

Company, respectively. Polyclonal rabbit anti-Tip60 antiserum was prepared after intradermal injection of New Zealand White rabbits with 1 mg of GST-Tip fusion protein in complete Freund's adjuvant.

Cell culture. 293 cells were maintained in DMEM medium (Gibco BRL) in the presence of 10% fetal calf serum (Hyclone), and 50 units/ml penicillin, 50 units/ml streptomycin (Gibco BRL). Characterization of TS-1 cells stably expressing different forms of Flag-tagged hIL-9Rα has been described previously (7).

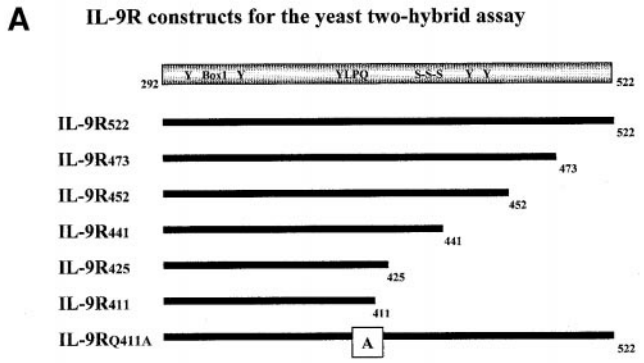
Yeast two-hybrid screen. A LexA-based yeast two-hybrid screening was performed as previously described (19). Briefly, L40 yeast containing the LexA-IL-9Rα intracellular domain and JAK1 kinase domain (pBTM-JAK-IL9R) was transformed with 500 μg plasmid DNA of a VP16 cDNA library derived from EML cells (20). The transformed cells were grown for 14 h at 30°C in liquid selective medium lacking uracil, tryptophan, and leucine to select for cotransformants and increase the efficiency of the *His*3 reporter gene expression. Cells were washed twice with THULL media (lacking tryptophan, histidine, uracil, leucine, and lysine) and plated in the same media. After 3 days at 30°C, colonies positive for X-Gal (21) staining were picked and grown in media lacking uracil and leucine but containing tryptophan to allow for loss of the bait-derived plasmid. The bait cured L40 yeast was mated with AMR70 yeast containing pBTM-JAK-IL9R, or pBTM-lamin by cocultivation. Mated yeast was spotted on THULL plates, and grown for 3–4 days at 30°C. Clones that grew and induced X-gal activity with the pBTM-JAK-IL9R but not with the pBTM-lamin constructs, were considered positive for the interaction with hIL-9Rα intracellular domain and were analyzed further. Yeast DNAs from positive clones were then used to transform bacterial strain DH5α. Plasmid DNAs from DH5α transformants were digested with *Not*I, and grouped according to restriction enzyme digestion patterns. Representative clones were then sequenced and DNA sequencing data analyzed by BLAST program. The yeast strain L40 carrying different hIL-9Rα constructs were transformed with pVP16-Tip (identified by yeast two-hybrid screening), plated on the THULL plates and grown for 3 days at 30°C. β-galactosidase assays in solution were performed as described previously (22), and the units of β-galactosidase activity were calculated by the Miller method (23).

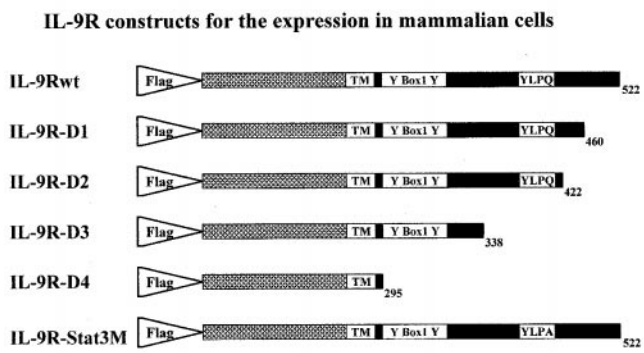
Plasmid construction. JAK1 kinase domain (amino acids 865–1142) was amplified by PCR, and subcloned into pAD4 vector (24) at HindIII site such that JAK1 expression is driven by *ADH1* promoter. The kinase expression cassette was then subcloned into PvuII site of pBTM116 to produce pBTM-JAK. Intracellular domain of hIL-9Rα (amino acids 292–522) was amplified by PCR, and subcloned into SalI site of pBTM-JAK to generate pBTM-JAK-IL9R, or subcloned into SalI site of pBTM116 to generate pBTM-IL9R522.

Plasmids used for yeast two-hybrid assay were prepared by subcloning PCR amplified truncated hIL-9Rα intracellular domains into EcoRI/BamHI sites of pBTM-116 to generate pBTM-IL9R411 (amino acids 292–411), pBTM-IL9R425 (amino acids 292–425), pBTM-IL9R441 (amino acids 292–441), pBTM-IL9R452 (amino acids 292–452), pBTM-IL9R473 (amino acids 292–473), pBTM-IL9R509 (amino acids 292–509) and pBTM-IL9RQ409A (amino acids 292–522, where Glu 409 was mutated to Ala). The construction and characterization of pFlagIL-9Rwt (amino acids 1–522), pFlagIL-9R-D1 (amino acids 1–460), pFlagIL-9R-D2 (amino acids 1–422), pFlagIL-9R-D3 (amino acids 1–338), pFlagIL-9R-D4 (amino acids 1–295), and pFlagIL-9R-Stat3M (amino acids 1–522, where Glu 409 was mutated to Ala), have been described previously (7). pFlag-mIL-4R was obtained from Genetics Institute (Cambridge, MA). pMTmyc-Tip60 was prepared by subcloning Tip60 cDNA into EcoRI site of pMTmyc vector. Subcloning of Tip60 into pGEX-2T, and preparation and purification of GST-Tip fusion proteins have been described previously (25).

Transient cell transfection, immunoprecipitation and Western blot analysis. 293 cells (2×10^6) were transfected by calcium phosphate precipitation method (26) with various plasmid combinations as indicated. Forty-eight hours later cells were washed with PBS and 1 ml ice-cold lysis buffer (LB) [50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1%

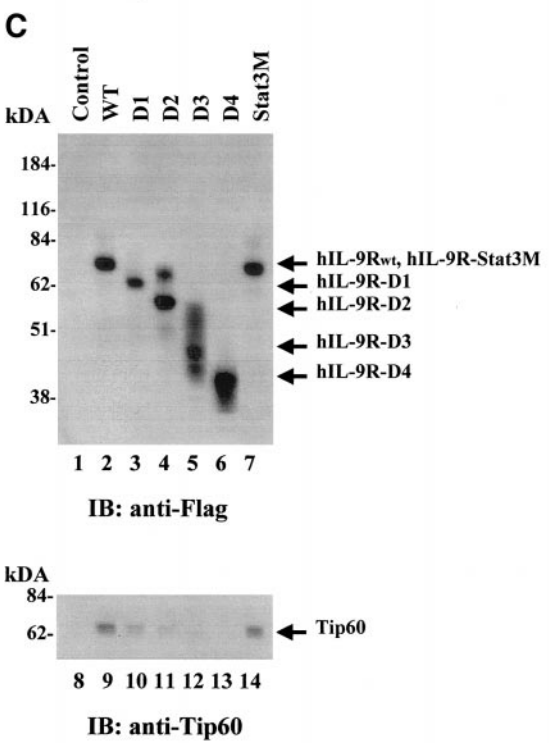
FIG. 2. Tip60 binds to amino acids 411–423 in hIL-9Rα. (A) Schematic representation of hIL-9Rα constructs used for protein–protein interaction in the yeast two-hybrid assay and expression in mammalian cells. (B) pBTM-LexA constructs with full-length, truncated or mutated intracellular domain of hIL-9Rα (pBTM-IL9R522, pBTM-IL9R509, pBTM-IL9R473, pBTM-IL9R452, pBTM-IL9R441, pBTM-IL9R425, pBTM-IL9R411, and pBTM-IL9RQ409A) were coexpressed with pVP16-Tip plasmid in L40 yeast. Transformants were assayed for β-galactosidase activity by the solution assay. β-Galactosidase activity is reported in Miller units ± S.D. using a minimum of three independent colonies. (C) 293 cells were cotransfected with 5 μg myc epitope tagged Tip60 (pMTmyc-Tip60), and 5 μg pFlag (control) (lanes 1, 8), or 5 μg pFlagIL-9Rwt (amino acids 1–522) (lanes 2, 9), or pFlagIL-9R-D1 (amino acids 1–460) (lanes 3, 10), or pFlagIL-9R-D2 (amino acids 1–422) (lanes 4, 11), or pFlagIL-9R-D3 (amino acids 1–338) (lanes 5, 12), or pFlagIL-9R-D4 (amino acids 1–295) (lanes 6, 13), or pFlagIL-9R-Stat3M (amino acids 1–522, where Glu 409 was mutated to Ala) (lanes 7, 14). After 48 h, cell lysates were prepared, and immunoprecipitated with anti-Flag antibodies. Immonoprecipitates were subjected to SDS-PAGE and immunobloted with anti-hIL-9R (lanes 1–7), and anti-Tip60 (lanes 8–14) antibodies, respectively. (D) 293 cells were cotransfected with 5 μg myc epitope tagged Tip60 (pMTmyc-Tip60), and 5 μg pFlag-mIL-4R. After 48 h, cell lysates were prepared, and immunoprecipitated with control rabbit IgG (lanes 1, 4), anti-Flag (lanes 2, 5), and anti-myc (lanes 3, 6) antibodies, respectively. Immunoprecipitates were subjected to SDS-PAGE and immunobloted with anti-Flag (lanes 1–3), and anti-myc (lanes 4–6) antibodies, respectively.

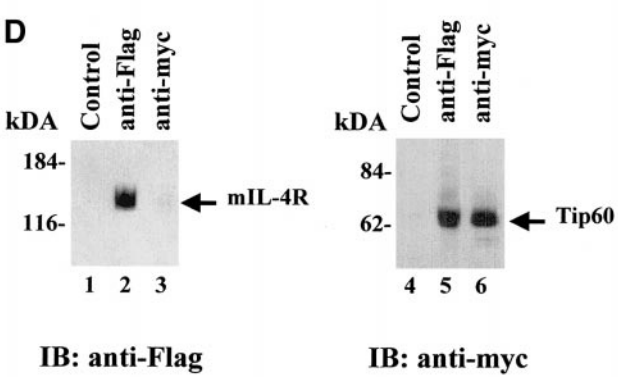




B

LexA-IL-9R constructs	β-Galactosidase Activity
IL-9R 292-522	28 +/- 7.1
IL-9R 292-473	29 +/- 11
IL-9R 292-452	33 +/- 15
IL-9R 292-441	21 +/- 5.8
IL-9R 292-425	31 +/- 6.6
IL-9R 292-411	3 +/- 2.1
IL-9R Q409A	81 +/- 21





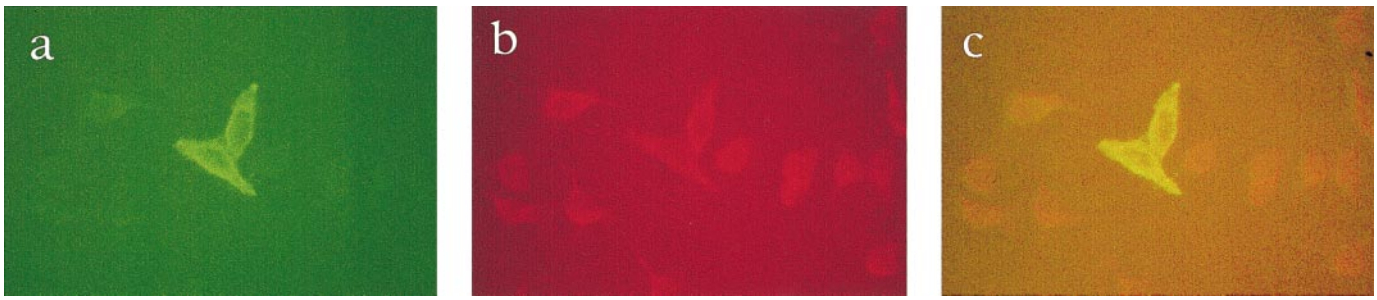


FIG. 3. Tip60 is expressed in cytoplasm and colocalized with hIL-9Rα. HeLa cells were transfected with 1 μg pFlagIL-9Rwt and 1 μg pMTmyc-Tip60. After 48 h the cells were incubated with anti-Flag and anti-Tip60 antibodies, and stained with fluorescein- (a, green) and rhodamine- (b, red) conjugated antibodies for the expression of hIL-9Rα and Tip60, respectively. Double image of a and b shows colocalization of hIL-9Rα and Tip60 (c, yellow). Detection of staining was performed by fluorescent microscopy.

NP-40, 1 mM EGTA, 100 μM Na_3VO_4, 15 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF] was added. Cells were lysed for 30 min at 4°C with occasional vortexing. The lysates were collected into Eppendorf tubes and cleared of nuclei by centrifugation for 10 min at 14,000 rpm. The supernatants (whole cell extracts) were immunoprecipitated with different antibodies as indicated for 16 h at 4°C with protein A-agarose beads added for the last two hours. The beads were then washed five times in buffer [20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 100 μM Na_3VO_4, 1 mM PMSF, 1 mM NaF]. Bound proteins were extracted with SDS-PAGE sample buffer, and analyzed by SDS-PAGE followed by Western blot with the ECL detection system.

Colocalization by immunofluorescence. HeLa cells were transfected by DOTAP (Boehringer Mannheim) according to the manufacturer's protocol with 1 μg each of pFlagIL-9Rwt and pMTmyc-Tip60. Two days after transfection, cells were washed with PBS, fixed with 3.7% formaldehyde for 15 min and permeabilized with 0.5% Triton X-100 for 5 min. After incubation for 3 h with anti-Flag M2 and anti-Tip60 antibodies (1:600), expression of hIL-9Rα was detected with anti-mouse fluorescein conjugated antibody (green), and expression of Tip60 with anti-rabbit rhodamine conjugated (red) antibody, by fluorescent microscopy.

RESULTS AND DISCUSSION

Binding of Tip60 to the Intracellular Domain of hIL-9Rα

In this study, we have identified Tip60 as one of the molecules that bind to the intracellular domain of the human interleukin-9 receptor α-chain (hIL-9Rα). We used a modified yeast two-hybrid screen, where kinase domain of JAK1 kinase is coexpressed with the LexA-IL-9Rα on a bait plasmid. We demonstrated previously that JAK1 autophosphorylated tyrosine as well as serine/threonine residues, and phosphorylation of hIL-9Rα is required for signaling (27). In addition, others (28, 29) have employed a modified yeast two-hybrid screen in which a kinase domain of PDGF receptor phosphorylated LexA-fusion proteins in the screening.

In order to confirm that the interaction between the intracellular domain of hIL-9Rα and Tip60 is phosphoprotein-dependent, we performed yeast two-hybrid assay using bait with (pBTM-JAK-IL9R) or without (pBTM-IL9R522) JAK1 kinase. Expression of JAK1 kinase increased the interaction between the intracellular domain of hIL-9Rα and Tip60 by about two fold (data not shown). It is possible that the interaction between hIL-9Rα and Tip60 resulted from the residues phosphorylated by endogenous yeast kinase activity, and expression of the kinase domain of JAK1 further increased the interaction between hIL-9Rα and Tip60.

IL-9 has previously been shown to stimulate phosphorylation of IL-9Rα (6). Therefore, we treated TS-1 cells stably expressing wild type Flag-tagged hIL-9Rα with human IL-9 for 5 minutes, and cell lysates were immunoprecipitated with monoclonal anti-Flag antibody. Immunoblot analysis with anti-hIL-9Rα, anti-phosphotyrosine, and anti-Tip60 antibodies showed that Tip60 binds to both non-phosphorylated and phosphorylated hIL-9Rα (Fig. 1). These results suggest that the interaction between IL-9Rα and Tip60 is independent of tyrosine phosphorylation status of IL-9Rα, and association between IL-9Rα and Tip60 does not require IL-9 stimulation.

Interaction of hIL-9Rα and Tip60 in Vivo

In an attempt to identify the minimal sequences of hIL-9Rα which are sufficient for interaction with Tip60, we constructed various mutants with truncated C-terminal regions of hIL-9Rα (Fig. 2A), and tested their interaction with pVP16-Tip in the yeast two-hybrid assay. Only pBTM-IL9R411 construct showed dramatic decrease in β-galactosidase activity, suggesting that the region between amino acids 411–423 in the hIL-9Rα is crucial for the binding to Tip60 (Fig. 2B). This region of hIL-9Rα is adjacent to the STAT3 binding motif whose mutation decreased IL-9 induced tyrosine phosphorylation of Stat3 (7). In order to test if Tip60 could prevent the binding of Stat3 to hIL-9Rα,

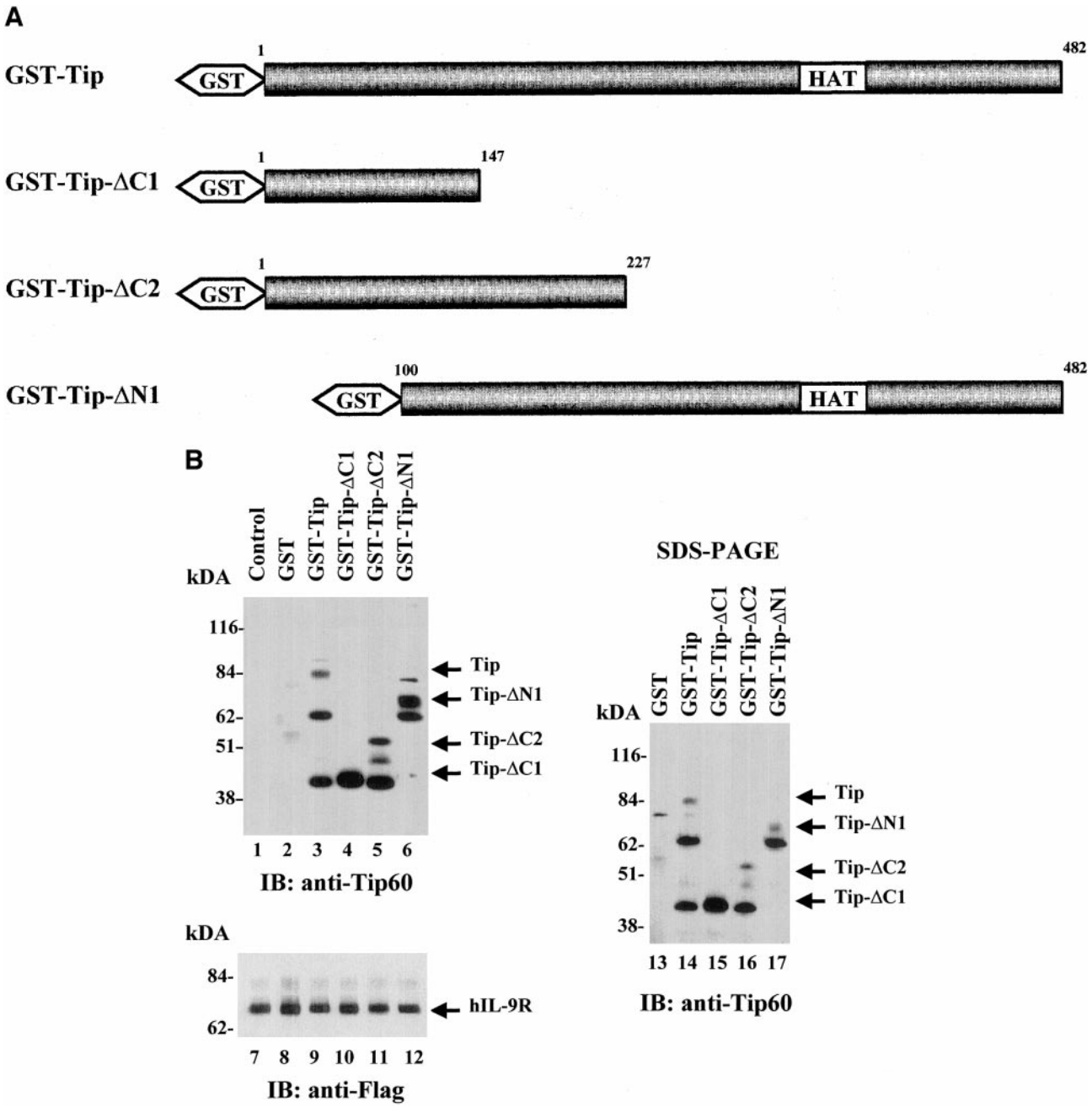


FIG. 4. hIL-9Rα binds to amino acids 100–147 in Tip60. (A) Schematic representation of GST-Tip fusion proteins (HAT-histone acetylatransferase domain). (B) 293 cells were transfected with 5 μg Flag epitope-tagged hIL-9Rα (pFlagIL-9Rwt). Whole cell lysates were incubated with control bacterial lysate (lanes 1, 7), GST protein (lanes 2, 8), GST-Tip fusion protein (1–482, 84 kDa) (lanes 3, 9), GST-Tip-ΔC1 fusion protein (1–147, 43 kDa) (lanes 4, 10), GST-Tip-ΔC2 (1–227, 53 kDa) (lanes 5, 11), and GST-Tip-ΔN1 (100–482, 68 kDa) (lanes 6, 12), and immunoprecipitated with anti-Flag antibodies. Immunoprecipitates were subjected to SDS-PAGE and immunobloted with anti-Tip60 (lanes 1–6), and anti-Flag (lanes 7–12) antibodies, respectively. 25 μl of expressed GST (lane 13), GST-Tip (lane 14), GST-Tip-ΔC1 (lane 15), GST-Tip-ΔC2 (lane 16), and GST-Tip-ΔN1 (lane 17) were subjected to 10% SDS-PAGE and immunobloted with anti-Tip60 antibodies.

lysates from 293 cells overexpressing Flag epitope-tagged hIL-9Rα, myc epitope-tagged Tip60, and HA epitope-tagged Stat3 were immunoprecipitated with monoclonal anti-Flag or anti-myc antibodies. Western blot analysis with anti-hIL-9Rα and anti-Tip60 antibodies revealed that overexpression of Stat3 did not affect the interaction between hIL-9Rα and Tip60 (data not shown).

To confirm the interaction between hIL-9Rα and Tip60 in mammalian cells, 293 cells were cotransfected

with Flag epitope-tagged hIL-9Rα expression vectors (pFlagIL-9Rwt, pFlagIL-9R-D1, pFlagIL-9R-D2, pFlagIL-9R-D3, pFlagIL-9R-D4 or pFlagIL-9R-Stat3M) (Fig. 2A), and a myc epitope-tagged Tip60 expression vector (pMTmyc-Tip60). After 48 h, cells lysates were prepared and immunoprecipitated with anti-Flag monoclonal antibody. Immunoblot analysis with anti-hIL-9Rα and anti-myc antibodies showed that Tip60 associates with IL-9Rwt, IL-9R-D1, IL-9R-D2, and IL-9R-Stat3M (Fig. 2C). In agreement with yeast two-hybrid analysis (Fig. 2B), IL-9R-D3 (amino acids 1–338) and -D4 (amino acids 1–295) did not interact with Tip60.

In order to test whether the binding of Tip60 is specific for hIL-9Rα, we cotransfected 293 cells with a Flag epitope-tagged mIL-4Rα and a myc epitope-tagged Tip60 expression vector. The intracellular domain IL-4Rα critical for signal transduction has been shown to be highly conserved between mouse and human receptors (30). Cell lysates were immunoprecipitated with control, anti-Flag, or anti-myc monoclonal antibodies. Immunoblot analysis with anti-Flag and anti-myc antibodies showed that mIL-4Rα and Tip60 also coimmunoprecipitated in cells (Fig. 2D). Therefore, the binding of Tip60 is not restricted to hIL-9Rα and Tip60 also binds to mIL-4Rα. Based on this observation, we speculate that biological specificity of IL-4 and IL-9 may be in part determined by different Stat transcription factors (Stat3 for IL-9 and Stat6 for IL-4) and both Stat3 and Stat6 may utilize Tip60 as a transcription cofactor. Interestingly, Tip60 has been recently identified as a coactivator for members within the nuclear hormone receptor superfamily which are ligand-activated and sequence specific transcription factors (30).

Colocalization of hIL-9Rα and Tip60

Since Tip60 was originally described as a nuclear protein (18; J. Kamine, unpublished results), our finding that Tip60 associates with membrane localized receptors such as IL-4Rα or IL-9Rα was rather surprising. In order to test whether Tip60 is also expressed in cytoplasm and binds to hIL-9Rα, we cotransfected HeLa cells with pFlagIL-9Rwt and pMTmyc-Tip60. Two days after transfection the expression of IL-9Rα was detected by fluorescent microscopy with fluorescein conjugated antibody (green), and expression of Tip60 with rhodamine conjugated (red) antibody (Fig. 3a,b). We detected both nuclear and cytoplasmic expression of Tip60. Double immunofluorescence showed that hIL-9Rα and Tip60 are colocalized (Fig. 3c, yellow). The colocalization of hIL-9Rα and Tip60 suggests that Tip60 may act as an adaptor molecule by linking different signaling molecules to cytokine receptor complexes.

Amino Acids 100–147 of Tip60 Are Necessary for Binding to hIL-9Rα

In order to identify which domain of Tip60 is necessary for binding to hIL-9Rα we performed GST pull-down experiments. Total cell lysates from 293 cells overexpressing hIL-9Rα were incubated with GST protein or truncated GST-fused Tip60 proteins (Fig. 4A), and immunoprecipitated with anti-Flag monoclonal antibody. Immunoblot analysis with anti-hIL-9Rα and anti-Tip60 antibodies showed that hIL-9Rα binds to the wild type GST-Tip (amino acids 1–482, 84 kDa), GST-Tip-ΔC1 (amino acids 1–147, 43 kDa), GST-Tip-ΔC2 (amino acids 1–227, 53 kDa), and GST-Tip-ΔN1 (amino acids 100–482, 68 kDa) (Fig. 4B). Therefore, the crucial Tip60 binding region is located between amino acids 100–147. This region is different from the LXXLL motif in the C-terminal domain of Tip60 (amino acids 458–462) that is essential for hormone-dependent interaction between coactivators such as SRC-1, TIF-2, CBP/300 and nuclear receptors (32, 33). We have previously demonstrated histone acetylation activity in immunoprecipitates of Tip60 from 293 cells (data not shown). Since Tip60 binds to a region near Stat3 binding site in hIL-9Rα, we suggest that Tip60 may act as a transcription cofactor for Stat3 through acetylation of histone, Stat3 or other transcription factors.

In summary, we have found that (i) Tip60 associates with the intracellular hIL-9Rα chain and mIL-4Rα chain, (ii) tyrosine phosphorylation of hIL-9Rα is not necessary for Tip60 binding, (iii) region 411–423 in hIL-9Rα is necessary for Tip60 binding, (iv) Tip60 is expressed in cytoplasm and colocalized with hIL-9Rα, and (v) region 100–147 in Tip60 is crucial for binding to hIL-9Rα. Further studies are necessary to elucidate the physiological role of Tip60 association with hIL-9Rα in IL-9 signaling pathway.

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