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Eps8 在 LPS 誘發巨噬細胞活化過程中生理意義之探討

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Participation of p97^{Eps8} in Src-mediated Transformation*

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Histone acetylase and histone deacetylase are two crucial enzymes that determine the structure of chromatin, regulating gene expression. In this study, we observed that trichostatin A (TSA), a specific histone deacetylase inhibitor, could effectively inhibit the growth of v-Src-transformed (IV5) cells and abrogate their ability to form colonies in soft agar. Further analysis demonstrated that, although TSA reduced the expression of Eps8 in a dose- and time-dependent manner, both the protein expression and kinase activity of v-Src remained constant, and the abundance and phosphotyrosine levels of Src substrates, including cortactin, focal adhesion kinase, p130^{Cas}, paxillin, and Shc, were not altered. Notably, removal of TSA from the medium restored not only the expression of Eps8, but also cellular growth. Northern and reverse transcription-PCR analyses revealed the significant reduction of *eps8* transcripts in TSA-treated IV5 cells relative to control cells. When active Src-expressing chicken embryonic cells were forced to overexpress p97^{Eps8}, they became resistant to TSA-mediated anti-proliferation. Furthermore, using small interference RNA of *eps8*, we demonstrated the requirement for Eps8 in IV5 cell proliferation. Thus, our results highlight a critical role for p97^{Eps8} in TSA-exerted growth inhibition of v-Src-transformed cells.

Eucaryotic DNA is regularly packed into nucleosomes that are folded into higher chromatin fibers (1). Approximately 146 bp of DNA are wrapped around a histone octamer containing two molecules each of H2A, H2B, H3, and H4 (1). Along with histone H1 and linker DNA, the nucleosome core constitutes the fundamental repeating unit of chromatin. A variety of post-translational modifications of histones, including phosphorylation, acetylation, methylation, ADP-ribosylation, and ubiquitination, have been reported (2). Among them, histone acetylation is the best studied and occurs at specific lysine residues in the N termini of the core histone (2), and its steady-state balance *in vivo* is regulated by histone acetylase and histone deacetylase (3–5). Adding acidic acetyl groups to lysine residues neutralizes the basic nature of histones and decreases their affinity for DNA. Therefore, the altered nucleosomal conformation increases the accessibility of transcriptional regulators to chromatin templates. Because histones are the major components in "chromatin remolding," their acetylation may also affect other DNA-templated processes, including DNA replication, repair, recombination, and chromosome segregation (6).

Trichostatin A (TSA)[1] was first discovered as an antifungal agent (7). Later, it was characterized as a noncompetitive inhibitor of histone deacetylase (8). TSA can induce a variety of biological responses, including induction of differentiation, cell cycle arrest, and apoptosis (8). Although TSA reverses cellular transformation caused by *sis*, *ras*, and *src* oncogenes *in vitro* (9–11), it also possesses antitumor activity against breast cancer *in vivo* (12). In addition to its anti-carcinogenic effect, TSA also affects angiogenesis (13) and fibrogenesis (14).

Eps8 is a substrate for both receptor and non-receptor tyrosine kinases (15–17). Two Eps8 isoforms (p97^{Eps8} and p68^{Eps8}) can be recognized by anti-Eps8 antibody (15). Although p68^{Eps8} has been speculated to be a proteolytic or an alternatively spliced product of p97^{Eps8}, its exact coding sequences and function are still unclear. Aberrant overexpression of p97^{Eps8} in cells not only enhances mitogenic responsiveness to epidermal growth factor (18), but also causes cellular transformation (19). Like various intracellular signal transducers, p97^{Eps8} contains several protein-protein interaction modules, including an SH3 domain, a putative nuclear targeting sequence, a split PH domain, a degenerated SH2 domain, and several proline-rich regions (15, 20). Through these domains, p97^{Eps8} has been reported to associate with a variety of signaling proteins such as Shc (21), Shb (22), RN-tre (23), and E3b1 (24). In addition, the split PH domain confers to p97^{Eps8} the ability to associate with the plasma membrane in response to serum stimulation, which is crucial for p97^{Eps8}-induced transformation (19). Recent accumulating evidence indicates that, by interacting with E3b1 and RN-tre, p97^{Eps8} is involved in the control of Rac and Rab5 signaling, respectively (25, 26). Reflecting its crucial role in signal transduction, the amount of p97^{Eps8} is modulated by growth and differentiation (16).

In this study, we demonstrate a strong correlation between TSA-mediated anti-carcinogenesis and the reduced expression of Eps8, which could be attributable to diminished *eps8* transcript levels in v-Src-transformed cells. Remarkably, small interference RNA (siRNA) of *eps8* significantly inhibited proliferation of v-Src-transformed cells, and ectopically expressed p97^{Eps8} imparted TSA resistance to cells expressing active Src. These results not only confirm p97^{Eps8} as an oncoprotein involved in v-Src-mediated transformation, but also reveal that its expression is TSA-sensitive.

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[1] The abbreviations used are: TSA, trichostatin A; SH, Src homology; PH, pleckstrin homology; siRNA, small interference RNA; CE, chicken embryonic; FAK, focal adhesion kinase; MOPS, 4-morpholinepropanesulfonic acid.

EXPERIMENTAL PROCEDURES

Cells and Lysate Preparation—The v-Src-expressing C3H10T1/2 (IV5) cells were a generous gift from Dr. Sarah J. Parsons (27). Chicken embryonic (CE) cells expressing mock control, active Src (c-Src 518 amber mutation), and focal adhesion kinase (FAK) or p97^{Eps8} in the active Src background were generated as described previously (17). Temperature-sensitive v-Src-expressing Rat-1 (R1/LA29) cells were kindly provided by Dr. Michael J. Weber (28). Cells grown to near-confluence were lysed in modified radioimmune precipitation assay buffer as described (29), and protein concentration was determined using the Bio-Rad protein assay kit.

Materials—TSA was purchased from Sigma. Antibody against Eps8 was either homemade (C-Eps8) (17) or purchased from Transduction Laboratories. Anti-FAK polyclonal antibody directed against the C-terminal region of FAK was described previously (30). Anti-Src (GD11) and anti-cortactin (4F11) antibodies were provided by Dr. Sarah J. Parsons. Antibodies recognizing p21$^{WAF1/CIP1}$, histone H3, acetylated histone H3, and phospho-Tyr^{317} Shc were obtained from Upstate Biotechnology, Inc. (Lack Placid, NY), and antibodies against Shc, p130^{Cas}, and paxillin were purchased from Transduction Laboratories. Horseradish peroxidase-conjugated mouse monoclonal antibody PY20 was purchased from Santa Cruz Biotechnology.

TSA Treatment and Cell Proliferation—IV5 and CE cells and CE cells expressing mock control, active Src, and FAK or p97^{Eps8} in the active Src background were incubated with or without various concentrations of TSA for different periods of time. Cells were washed, trypsinized, and counted using a hemocytometer.

Immunoprecipitation, Immunoblotting, and in Vitro Kinase Reaction—Immunoprecipitation of cortactin, FAK, Shc, p130^{Cas}, and paxillin was performed with their respective antibodies as described previously (31). For analysis of Tyr(P), cortactin, Eps8, FAK, Shc, phospho-Tyr^{317} Shc, p130^{Cas}, paxillin and Src, Western immunoblotting was performed with their respective antibodies, and detection was by enhanced chemiluminescence (Amersham Biosciences). The v-Src immunocomplex kinase reaction was carried out as described previously (17).

Analysis of Histone Acetylation—IV5 cells that had been treated with or without TSA were suspended in lysis buffer (10 mM Tris-HCl (pH 6.5), 50 mM sodium bisulfite, 1% Triton X-100, 10 mM $MgCl_2$, and 8.6% sucrose) and lysed in a Dounce homogenizer. The nuclei were collected by centrifugation and suspended in water. Histones were extracted with 0.4 N H_2SO_4, and acid-soluble histones were precipitated with 20% trichloroacetic acid. Histones were resolved by SDS-PAGE and transferred to nitrocellulose membrane for Western blot analysis. Total and acetylated histones H3 were detected with their respective antibodies.

Anchorage-independent Growth—Approximately 1500 cells were suspended in 0.5% agarose-containing Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum and various amounts of TSA and layered over an agarose plug on a 6-well plate. The agarose plug contained 1% agarose in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were incubated for 2 weeks, during which time fresh medium and TSA were added to the plates every 3 days. Colonies composed of more than eight cells were counted after 14 days.

Northern Analysis—Total cellular RNA was harvested by acidic phenol extraction (RNAtrap®, Protech) as suggested by the manufacturer and quantified using a spectrophotometer. RNA (20 μg) was loaded onto a 1% agarose gel cast in 20 mM MOPS (pH 7.0), 5 mM NaOAc, and 1 mM EDTA containing 0.6% formaldehyde. RNA was transferred onto a nylon membrane by capillary blotting and fixed by UV cross-linking. The mouse *eps8*-containing full-length open reading frame or β-actin cDNA probes (prepared as described under "Reverse Transcription-PCR") were labeled with [α-^{32}P]dCTP. The blots were washed three times at room temperature for 10 min with 2× SSC and 1% SDS, twice at 60 °C for 20 min with 1× SSC and 0.1% SDS, and twice at room temperature for 10 min with 0.1× SSC and 0.1% SDS.

Reverse Transcription-PCR—Total RNA was isolated as described above. Approximately 5 μg of total RNA was reverse-transcribed into single-stranded cDNA in a 50-μl reaction mixture containing 10 μl of 5× reaction buffer, 60 pmol of oligo(dT)$_{15}$, 40 units of RNase inhibitor, and 200 units of Moloney murine leukemia virus reverse transcriptase (Promega). The reaction was carried out at 42 °C for 1 h. Single-stranded cDNA products (1 μl) were then amplified by PCR. The PCR was carried out in a 50-μl mixture containing 5 μl of 10× reaction buffer, 0.2 mM dNTP, 0.2 μM forward and reverse primers for *eps8* encoding p97^{Eps8}, and 2.5 units of *Taq* polymerase using an Applied Biosystems GeneAmp PCR System 2400. As an internal standard, a pair of primers for β-actin was included at the same time. The PCR was carried out according to the following program. The cDNA was denatured for 5 min at 94 °C and amplified for 30 cycles under optimal conditions: 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, followed by a 5-min elongation step at 72 °C. Different numbers of cycles were tested to ensure linear phase amplification of the cDNA. The sequences of primer pairs used were as follows: p97^{Eps8}, 5′-ATGAATGGTCATATGTCTAA-3′ (sense) and 5′-CTGTGTGACAGTCCCCGGTG-3′ (antisense); and β-actin, 5′-ATCATGTTTGAGACCTTCAA-3′ (sense) and 5′-CATCTCCTGCTCGAAGTCTA-3′ (antisense). The PCR products were resolved on a 1% agarose gel and detected by ethidium bromide staining.

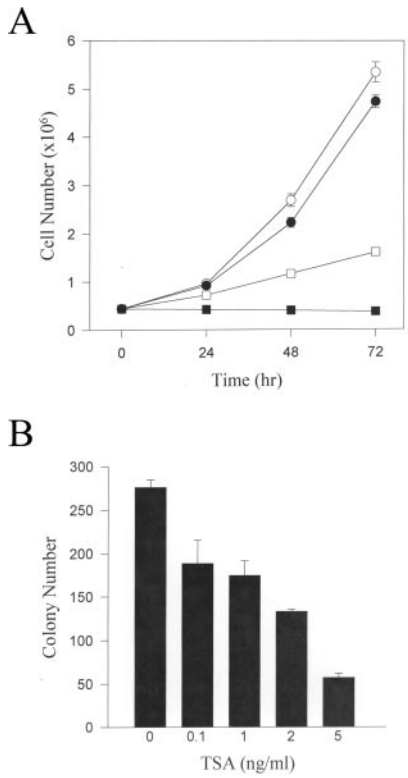

FIG. 1. **Inhibitory effect of TSA on the growth and transformation of v-Src-transformed cells.** *A*, cells expressing v-Src (IV5 cells) were plated at a density of 3×10^5 cells/60-mm dish. After 18 h (time 0), different concentrations of TSA (0 (○), 1 (●), 10 (□), and 100 (■) ng/ml) were added to the medium and incubated with the cells for 24, 48, and 72 h. The total numbers of control and TSA-treated cells were counted and plotted. The results are shown as means ± S.D. of triplicate experiments. Similar results were obtained at least three times. A representative experiment is shown. *B*, IV5 cells (1.5×10^3/plate) were seeded in triplicate and incubated with various concentrations of TSA as indicated for 2 weeks. The values for the number of colonies are means ± S.D. of three experiments.

Construction and Transfection of eps8 siRNA—To construct a plasmid expressing *eps8* siRNA, two complementary *eps8*-containing oligonucleotides (sense strand, 5′-GATCCCGCAGCAGCTCCAGTGACAGTTTCAAGAGAACTGTCACTGGAGCTGCTGTTTTTTGGAAA-3′; and antisense strand, 5′-AGCTTTTCCAAAAAACAGCAGCTCCAGTGACAGTTCTCTTGAAACTGTCACTGGAGCTGCTGCGG-3′) were synthesized, annealed, and ligated into the p*Silencer* hygro vector (Ambion Inc., Austin, TX) according to the manufacturer's recommendation. To generate IV5 cells expressing *eps8* siRNA or the nonspecific negative control, p*Silencer*-Eps8 or the negative control p*Silencer* hygro vector, respectively, was transfected into IV5 cells using LipofectAMINE Plus (Invitrogen) following the method recommended by the manufacturer. These cells were then cloned by hygromycin (400 μg/ml) selection for ~10 days.

RESULTS

TSA Inhibits the Proliferation and Transformation of Cells Expressing v-Src—Previous studies have indicated that TSA can reverse transformed phenotypes caused by multiple oncogenes (9–11). In an attempt to study the influence of TSA on

the cellular growth of v-Src-transformed (IV5) cells, various concentrations of TSA (0, 1, 10, and 100 ng/ml) were applied to IV5 cells for different periods of time, and their proliferation was assessed. As shown in Fig. 1*A*, whereas 1 ng/ml TSA exerted a slightly inhibitory effect, 10 ng/ml TSA significantly caused growth inhibition of IV5 cells after a 48-h exposure. When the TSA concentration reached 100 ng/ml, a complete block of cellular growth was detected. With respect to transformation, ~2 ng/ml TSA could repress 50% colony formation of IV5 cells in soft agar (Fig. 1*B*). These results indicate that TSA is an effective growth and transformation inhibitor for cells expressing v-Src.

TSA Induces Histone Acetylation in v-Src-transformed Cells—TSA is a well known histone deacetylase inhibitor that can augment histone acetylation. To confirm its effect in IV5 cells, cells were cultured with or without 100 ng/ml TSA for various periods of time, and the histones were isolated. Western blot analysis demonstrated that, prior to incubation with TSA (0 h), the levels of acetylated histone H3 in IV5 cells were low (Fig. 2). Incubation with TSA resulted in the accumulation of acetylated histone H3, the level of which then remained constant over a 36-h incubation period. Interestingly, unlike previously documented up-regulation (32, 33), the level of the cyclin-dependent kinase inhibitor p21$^{WAF1/CIP1}$ was TSA-insensitive in IV5 cells (Fig. 2).

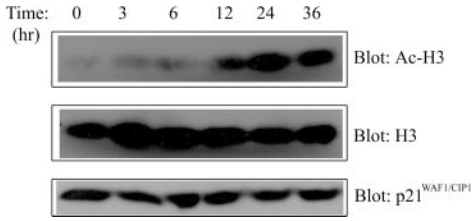

FIG. 2. **TSA causes accumulation of acetylated histone H3 in v-Src-transformed cells.** IV5 cells were treated with 100 ng/ml TSA for the indicated times. Cell lysates or histones were isolated as described under "Experimental Procedures." Equal amounts of histones (30 μg; *upper* and *middle panels*) and lysates (100 μg; *lower panel*) were separated by SDS-PAGE and analyzed by Western immunoblotting with anti-acetylated histone H3 (*Ac-H3*), anti-histone H3, and anti-p21$^{WAF1/CIP1}$ antibodies, respectively.

TSA Treatment Does Not Affect v-Src Enzymatic Activity—As shown above, we have demonstrated that TSA can effectively inhibit v-Src-mediated transformation. Because v-Src is a tyrosine kinase and because its activity plays an important role in oncogenesis, we investigated the influence of various concentrations of TSA on the tyrosyl phosphorylation of proteins in IV5 cells after a 24-h treatment. As demonstrated in Fig. 3*A*, no significant alteration in the amounts of v-Src along with the Tyr(P) content of a number of cellular proteins occurred in TSA-exposed IV5 cells, suggesting that TSA did not alter v-Src expression and its kinase activity. To further confirm this point, an *in vitro* kinase assay using the Src immunoprecipitates from IV5 cells exposed to various concentrations of TSA was performed. According to Fig. 3*B*, TSA did not alter the activity of Src even when its concentration reached 200 ng/ml. Consistently, no significant changes in the Tyr(P) residues of Src potential substrates, including cortactin, FAK, p130^{Cas}, paxillin, and phospho-Tyr^{317} Shc, were detected (Fig. 3*C*). These data show that the activity of v-Src remained constant in TSA-treated IV5 cells.

Down-regulation of p97^{Eps8} in TSA-treated v-Src-transformed Cells—Because p97^{Eps8} was previously demonstrated to be an oncoprotein whose expression is enhanced in v-Src-transformed cells (17), we were interested to know whether the TSA-induced antitumorigenic effects in IV5 cells are mediated through p97^{Eps8}. To address this issue, the levels of p97^{Eps8} in IV5 cells incubated with or without various concentration of TSA for 24 h were analyzed. As demonstrated in the dose-response experiment (Fig. 4*A*), in agreement with growth inhibition by TSA, an obvious reduction of Eps8 (both p97^{Eps8} and p68^{Eps8}) was detected in cells treated with 10 ng/ml TSA, whereas nearly complete abrogation of p97^{Eps8} was observed when the concentration of TSA reached 100 ng/ml. By contrast, the constant expression of the other Src substrates (*i.e.* cortactin, FAK, p130^{Cas}, paxillin, and Shc) in response to TSA in IV5 cells, implying the down-regulation of Eps8 by TSA, was quite specific (Fig. 3*C*). Furthermore, TSA reduced the expression of Eps8 in a time-dependent manner; and after 12–24 h of exposure to 100 ng/ml TSA, Eps8 expression almost totally vanished (Fig. 4*B*). In addition to IV5 cells, this TSA-suppressed

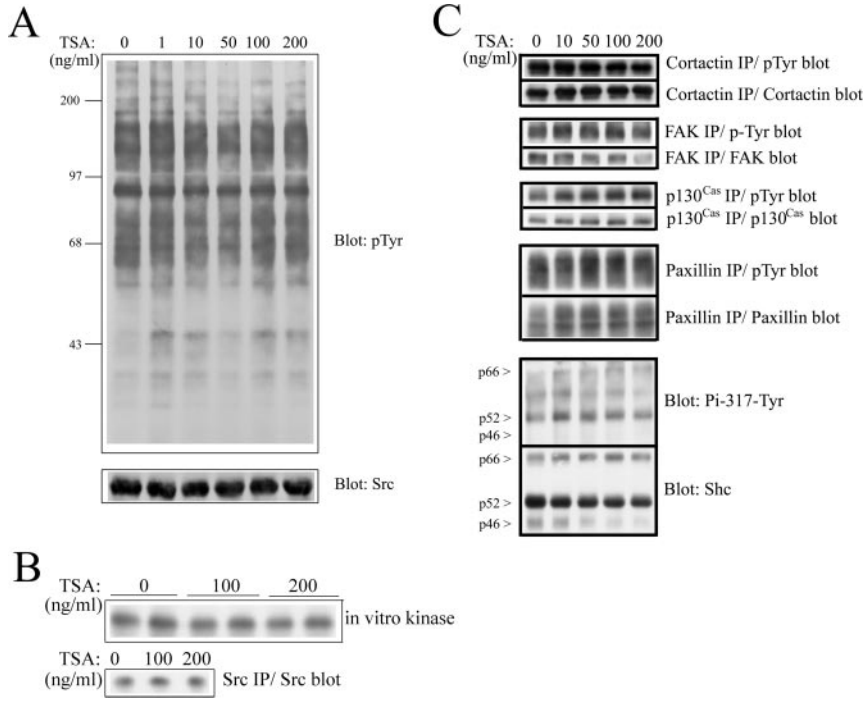

FIG. 3. **TSA exposure does not alter Src kinase activity in v-Src-transformed cells.** IV5 cells were treated with various concentration of TSA for 24 h. *A*, equal amounts of cell lysates (100 μg) were analyzed directly by Western immunoblotting with anti-Tyr(P) (*pTyr*; *upper panel*) and anti-Src (*lower panel*) antibodies. *B*, lysates (600 μg) prepared from non-treated or TSA-treated IV5 cells were immunoprecipitated (*IP*) with anti-Src antibody. One-third of the immunocomplexes were divided into two parts and subjected to *in vitro* kinase reaction in the presence of [γ-^{32}P]ATP. ^{32}P-Labeled Src was analyzed by SDS-PAGE and detected by autoradiography (*upper panel*). The rest of the immunocomplexes were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and probed with anti-Src antibody (*lower panel*). *C*, equal amounts of cell lysates (100 μg) were analyzed directly by Western immunoblotting with antibodies against Shc and phospho-Tyr^{317} Shc (*Pi-317-Tyr*). The cortactin, FAK, p130^{Cas}, and paxillin immunocomplexes prepared from each sample were analyzed by Western immunoblotting with anti-Tyr(P) antibody and their corresponding antibodies.

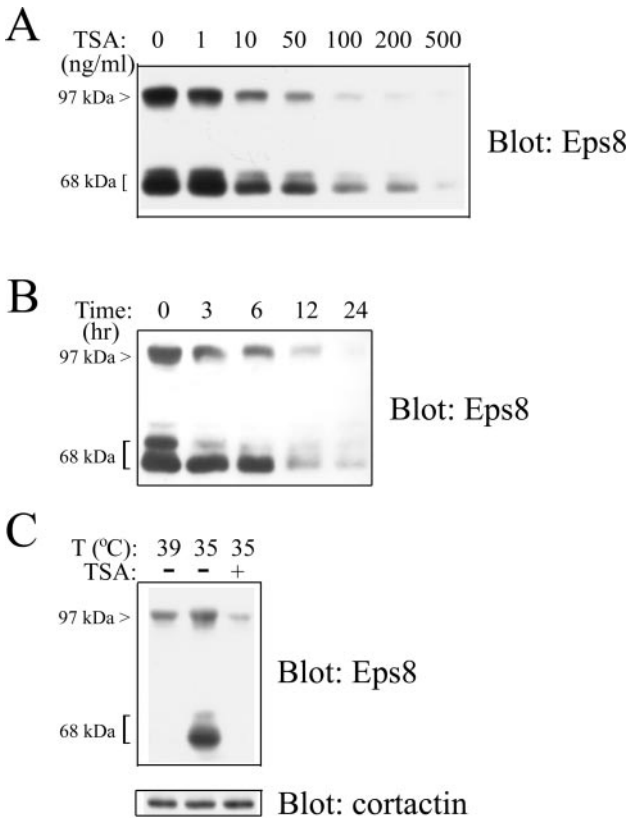

FIG. 4. **Reduced p97^{Eps8} expression in v-Src-transformed cells exposed to TSA.** *A*, shown is the dose-response curve of TSA-reduced p97^{Eps8}. Cells expressing v-Src (IV5 cells) were treated with various concentration of TSA for 24 h. Equal amounts of cell lysates (100 μg) were analyzed directly by Western immunoblotting with anti-Eps8 antibody. *B*, shown is the time-response curve of TSA-reduced p97^{Eps8}. IV5 cells were treated with TSA (100 ng/ml) for different periods of time. Cell lysates (100 μg) from each group were examined by direct Western immunoblot analysis for Eps8 as described for *A*. *C*, lysates from R1/LA29 cells incubated at 39 or 35 °C in the presence or absence of TSA were harvested. Equal amounts of lysates were analyzed directly by Western immunoblotting with anti-Eps8 (*upper panel*) and anti-cortactin (*lower panel*) antibodies.

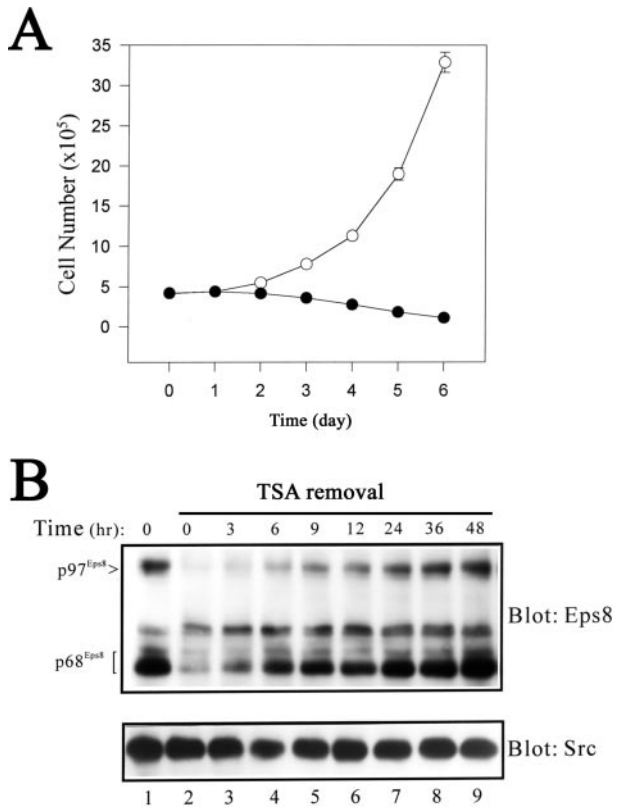

FIG. 5. **Removal of TSA restores the cellular growth and protein expression of p97^{Eps8}.** IV5 cells (3×10^5) were plated at the beginning of the experiment. After cellular attachment, cells were treated with 100 ng/ml TSA for 24 h and then incubated in medium with (●) or without (○) TSA for various lengths of time as indicated. *A*, the total number of cells in each group was counted and plotted. The results are shown as means ± S.D. of triplicate experiments. *B*, equal amounts of lysates from each group were analyzed directly by Western immunoblotting with C-Eps8 (*upper panel*) and anti-Src antibody (*lower panel*).

Eps8 expression was also observed in other C3H10T1/2-derived cell lines expressing v-Src (data not shown). Most important, similar phenomena could also be detected in R1/LA29 cells expressing temperature-sensitive v-Src (28). Whereas Eps8 could be induced by a temperature shift (39 to 35 °C) (28), its down-regulation could be achieved by TSA treatment (Fig. 4*C*). In contrast, consistent with our previous finding, the expression of cortactin was TSA-insensitive (Fig. 4*C*).

Removal of TSA Can Restore the Expression of p97^{Eps8} and Cellular Growth—Because TSA-inhibited cellular growth of v-Src-transformed cells coincided with the down-regulation of Eps8, we therefore speculated that TSA exerts its anti-mitogenic effect through Eps8 reduction. If this hypothesis were correct, TSA removal should restore Eps8 expression, which, in turn, results in resumption of v-Src-transformed cell proliferation. To prove this speculation, IV5 cells initially exposed to TSA (100 ng/ml) for 24 h were incubated with TSA-free medium. The growth curve and expression of Eps8 in these cells were then analyzed. As expected, TSA withdrawal resulted in the resumption of cellular growth as compared with its persistent presence (Fig. 5*A*). Interestingly, whereas a dramatic reduction of Eps8 was observed in 24-h TSA-treated cells, significantly increased Eps8 expression was detected in IV5 cells after 6-h TSA removal, and the longer the cells were incubated with TSA-free medium, the more Eps8 expression was restored (Fig. 5*B*). Concurrent with our previous finding that TSA did not modulate the expression of v-Src in IV5 cells, no appreciable alteration in v-Src expression after TSA removal was observed (Fig. 5*B*).

Reduced eps8 Transcript Levels in TSA-treated v-Src-transformed Cells—Two *eps8* transcripts (*i.e.* 4.7 and 3.8 kb) were reported previously (15). Characterization of the N terminus of p97^{Eps8} (amino acids 1–175) disclosed its absence in p68^{Eps8} (17). Analysis of its corresponding cDNA by Northern blotting revealed only the 4.7-kb transcript, which was believed to encode p97^{Eps8} (data not shown). Because TSA could down-regulate the expression of Eps8, we thereby wondered whether TSA could influence the abundance of *eps8* transcripts. To answer this question, total RNAs extracted from control and TSA-treated IV5 cells were analyzed by Northern blotting. As demonstrated in Fig. 6*A*, the abundance of both 4.7- and 3.8-kb *eps8* transcripts was significantly decreased in TSA-treated cells within 12 h. Further reverse transcription-PCR analysis utilizing specific primers for the 4.7-kb *eps8* transcript revealed that TSA treatment led to a decrease in the expected 678-bp product (Fig. 6*B*). These results imply that TSA-mediated decreases in p97^{Eps8} expression could be partly attributable to the reduction of its corresponding *eps8* transcript.

Ectopically Expressed p97^{Eps8} Confers Resistance to TSA—The strong correlation between TSA-mediated growth arrest and TSA-reduced p97^{Eps8} expression in IV5 cells prompted us to investigate the involvement of p97^{Eps8} in TSA-mediated anti-carcinogenesis in cells expressing v-Src. To address this

point, CE cells expressing barely detectable Eps8 levels were chosen as our model system (Ref. 17 and data not shown). CE cells ectopically expressing active Src or active Src along with p97^{Eps8} were incubated with or without TSA, and their mitogenesis in response to TSA was assessed. As demonstrated in Fig. 7*A*, whereas TSA (100 ng/ml) totally inhibited the cellular growth of active Src-expressing cells, overexpression of p97^{Eps8} almost completely reversed this inhibition. In comparison, overexpression of FAK did not alleviate this TSA-mediated growth arrest (Fig. 7*B*). These results indicate that p97^{Eps8} is indeed an important TSA target whose down-regulation contributes to the antitumorigenic activity of TSA in v-Src-transformed cells.

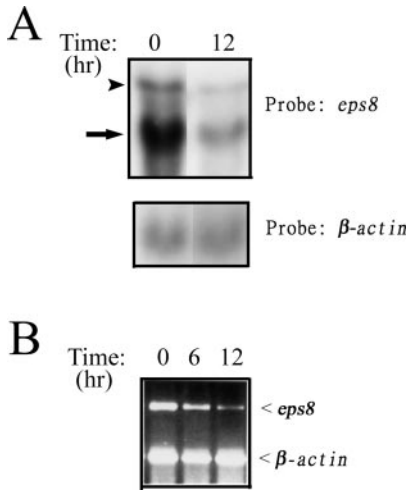

FIG. 6. **Reduced *eps8* transcript levels in response to TSA.** IV5 cells were left untreated or were treated with 100 ng/ml TSA for various times as indicated. *A*, total RNA (30 μg) extracted from these cells was examined by Northern analysis. Filters were hybridized with probes for *eps8* and β-actin. The 4.7- and 3.8-kb transcripts are indicated by the *arrowhead* and *arrow*, respectively. *B*, in this semiquantitative reverse transcription-PCR analysis, one-fiftieth of the total RNA-derived cDNA was used for PCR as described under "Experimental Procedures." β-Actin was utilized as an internal control for amplification efficiency.

Down-regulation of Eps8 Inhibits the Cellular Growth of v-Src-transformed Cells—The above results strongly suggested that Eps8 plays an essential role in IV5 cell proliferation. To substantiate this hypothesis, plasmids expressing nonspecific or *eps8*-specific siRNA were generated and introduced into IV5 cells. Whereas there was a >50% reduction of Eps8 expression (both 97- and 68-kDa proteins) in two selected IV5 cell lines constitutively expressing *eps8* siRNA (*i.e.* siRNA-1 and siRNA-2), the expression of Eps8 was unchanged in those cell lines expressing nonspecific siRNA (*i.e.* Ctrl-1 and Ctrl-2) compared with parental IV5 cells (Fig. 8*A*). Concomitantly, the proliferation of these cells was examined. As shown in Fig. 8*B*, in comparison with IV5, Ctrl-1, and Ctrl-2 cells, the cellular growth of siRNA-1 and siRNA-2 cells was suppressed. This finding implies the requirement for Eps8 in IV5 cell proliferation.

DISCUSSION

The level of histone acetylation is determined by equilibration between histone acetylase and histone deacetylase, which affects the dynamics of chromatin folding during gene transcription (34). Inhibitors of histone deacetylase such as *n*-butyrate and TSA were found to cause accumulation of hyperacetylated histone H4 within cells (35). A variety of biological responses, including cell cycle progression, differentiation, and apoptosis, are affected by TSA. In this study, we have demonstrated that TSA can effectively cause growth inhibition of v-Src-transformed cells and concomitantly down-regulate the expression of p97^{Eps8}. Interestingly, no alterations in the amounts and enzymatic activity of v-Src were detected in TSA-

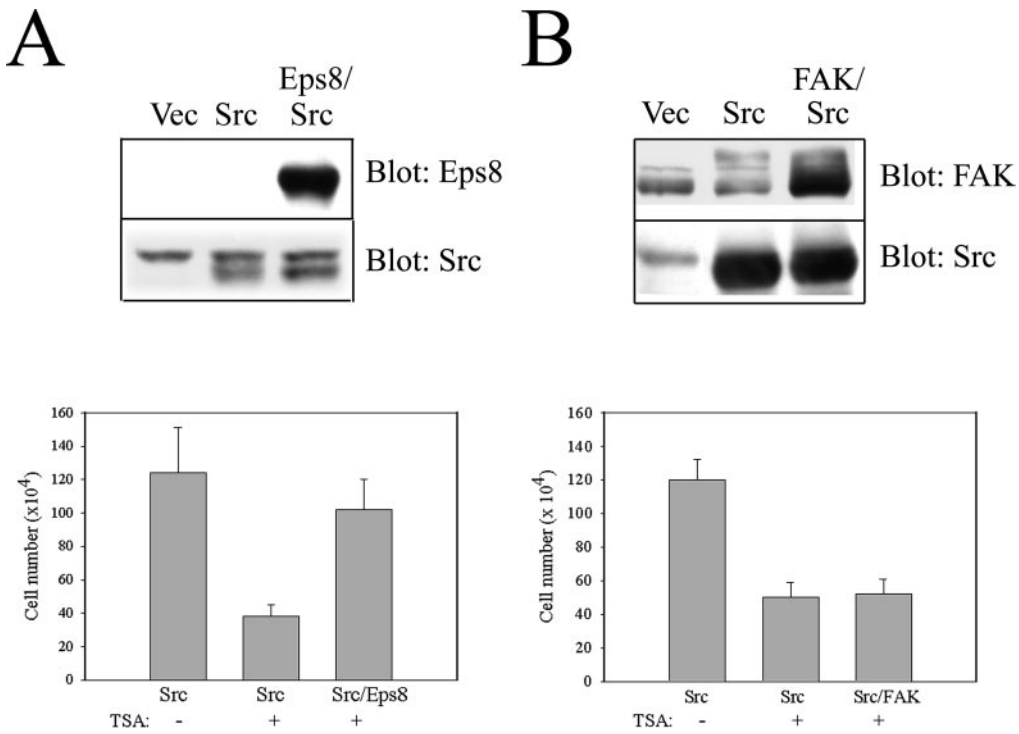

FIG. 7. **Cells ectopically expressing p97^{Eps8} resist TSA-induced growth arrest.** CE cells (5×10^5) expressing mock control (*Vec*), active Src (*Src*), both p97^{Eps8} and active Src (*Eps8*/*Src*), and both FAK and active Src (*FAK*/*Src*) are described under "Experimental Procedures." Equal amounts of cell lysates (100 μg) from each sample were resolved by SDS-PAGE and analyzed by Western immunoblotting with C-Eps8 or anti-Src antibody (*A*) and with anti-FAK or anti-Src antibody (*B*). Cells (~5×10^5) of interest were treated with or without 100 ng/ml TSA. After a 24-h incubation, cells in each treatment were harvested and counted. The results are shown as means ± S.D. of triplicate experiments. Similar results were obtained at least twice.

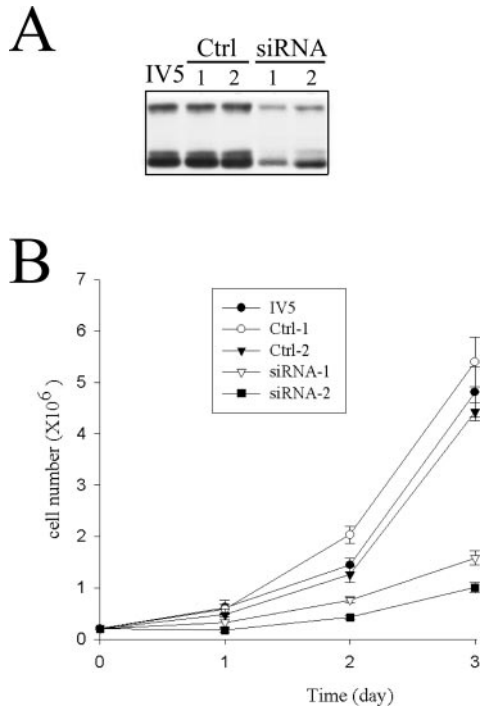

FIG. 8. ***eps8* siRNA reduces Eps8 expression in and proliferation of v-Src-transformed cells.** The generation of *eps8* siRNA expression cell lines (siRNA-1 and siRNA-2) and the negative control cell lines (Ctrl-1 and Ctrl-2) from IV5 cells is described under "Experimental Procedures." *A*, equal amounts of their lysates were analyzed directly by Western immunoblotting with anti-Eps8 antibody. *B*, the cells indicated were plated at a density of 3×10^5 cells/60-mm dish at the beginning of the experiment. After incubation for 1, 2, and 3 days, the total number of cells was counted and plotted. The results are shown as means ± S.D. of triplicates. Similar results were obtained three times.

treated v-Src-transformed cells, and withdrawal of TSA restored both p97^{Eps8} expression and cellular growth. The increased TSA resistance of cells overexpressing p97^{Eps8} further confirmed the importance of diminished p97^{Eps8} expression in the antitumorigenic effect exerted by TSA. As an extension, inhibition of the mitogenesis of v-Src-transformed cells by *eps8* siRNA further verified the requirement for Eps8 in IV5 cell proliferation.

Inhibition of the c-*src* promoter and repression of c-*src* mRNA as well as protein expression by TSA have been reported (36). This seems to be in conflict with our finding that TSA did not interfere with Src expression and its kinase activity in IV5 cells. However, these independent results are not contradictory. This is because the cells that we used were transfected with v-*src* cDNA, which is under the influence of the Moloney murine leukemia virus long terminal repeat instead of the cognate c-*src* promoter that retains TSA-sensitive regions (27).

Given that TSA is a well documented histone deacetylase inhibitor and that we observed significant reduction of *eps8* transcripts in TSA-treated IV5 cells, it is tempting to speculate that the TSA-mediated down-regulation of p97^{Eps8} is exerted at the transcriptional level because similar results were obtained when IV5 cells were exposed to butyrate, another well known histone deacetylase inhibitor (data not shown). Whereas acetylation of histone proteins might alter the chromatin structure and allow binding of transcription factors, acetylation of non-histone proteins such as transcription factors and components of the basal transcriptional machinery might affect their activity. Alternatively, acetylation of histone and non-histone proteins might create (or eliminate) a signal that is recognized by another factor(s). This is reminiscent of protein phosphorylation, which can either alter the conformation and function of a protein or provide the binding sites for effector molecules. These models may partially overlap and are not mutually exclusive. Nevertheless, we did not exclude the possibility that TSA treatment might decrease the stability of *eps8* mRNA and its translation product.

Although p97^{Eps8} has been verified as an oncoprotein, the biological significance of its augmented expression in v-Src-transformed cells (17, 19) is still obscure. The suppression of IV5 cell proliferation by *eps8* siRNA unveiled the importance of Eps8 in transmitting growth signals in v-Src-transformed cells. Our observation that active Src-expressing CE cells with ectopically expressed p97^{Eps8} resisted TSA-mediated anti-proliferation substantiates this notion. It is noteworthy that, for reasons unknown, we were unable to obtain high ectopic p97^{Eps8} expressers from IV5 cells that already had substantially high amounts of Eps8. Therefore, CE cells expressing barely detectable Eps8 levels (Fig. 7*A* and data not shown) were chosen as our alternative to study p97^{Eps8}-conferred TSA resistance. Remarkably, unlike p97^{Eps8}, CE cells ectopically expressing FAK in the background of active Src did not become TSA-resistant (Fig. 7*B*). This is consistent with unaltered FAK expression in TSA-treated IV5 cells (Fig. 4*C*) and implies the specificity of TSA action. Intriguingly, in addition to p97^{Eps8}, the expression of p68^{Eps8} was also down-regulated in response to TSA, in essential agreement with the reduced abundance of *eps8* transcripts observed in our Northern blot analysis (Fig. 6*A*). Because the identity and biological function of p68^{Eps8} are still unknown, the implication of its down-regulation in TSA-mediated growth arrest of v-Src-transformed cells is unclear at this moment.

To date, TSA has been shown to modulate the expression of a spectrum of genes. It has been reported that, like p97^{Eps8}, the down-regulation of Arp2 (actin-related protein-2), Arp3, and RhoA (37) and syndecan-2 (38) correlates with impaired migration in hepatic stellate cells and reduced tumorigenic activity in colon carcinoma cells. In contrast, a variety of proteins can be up-regulated by TSA. The prominent examples include p21$^{WAF1/CIP1}$ (32, 33), gelsolin (35), cytokeratin A (*endoA*) (39), Stra13 (40), and plasminogen activator (41). p21$^{WAF1/CIP1}$ is an inhibitor of cyclin-dependent kinase, and Stra13 is a transcriptional repressor of the c-*myc* gene. Thus, by up-regulation of p21$^{WAF1/CIP1}$ and Stra13, TSA could cause cell cycle arrest and growth inhibition, respectively. However, we did not observe TSA-mediated p21$^{WAF1/CIP1}$ induction in IV5 cells (Fig. 2). This discrepancy is likely due to the different cells studied in various systems. It is particularly noteworthy that *eps8* is not among the TSA-responsive genes identified by microarrays (42). Considering the role of *eps8* in transformation and its sensitivity to TSA treatment, our findings might supplement and expand our understanding of the mechanisms by which TSA exerts its anti-proliferation in cancer cells.

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