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Expression of Transcriptional Repressor Protein mSin3A But Not mSin3B Is Induced during Neuronal Apoptosis

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mSin3 proteins have an important role in transcriptional repression mediated by histone deacetylation. Our purpose was to find out whether apoptosis affects the expression of mSin3 proteins in neuroblastoma 2a cells. We observed that neuronal apoptosis, induced by serum withdrawal or by treatment with etoposide, okadaic acid or trichostatin A, induced a prominent increase in mSin3A protein expression but did not affect the level of mSin3B protein. Trichostatin A, an inhibitor of histone deacetylases, induced the most prominent upregulation of mSin3A protein. Metabolic labeling and immunoprecipitation of mSin3A showed a marked increase in the synthesis of mSin3A protein in agreement with the immunoblotting results. Interestingly, the expression of mSin3A preceded the activation of caspase-3 and the execution phase of neuronal apoptosis. These results suggest that the expression of mSin3A proteins may provide a regulation mechanism to enhance transcriptional repression or silencing of genes during neuronal apoptosis, as well as during degenerative diseases. © 1998 Academic Press

Recent studies have shown that histone acetylation has an important role in transcriptional regulation (1, 2). Several transcription factors bind co-activators, such as CREB-binding protein (p300/CBP) or P/CAF, which are histone acetyltransferases and enhance nucleosomal relaxation and activation of transcription (2-4). On the contrary, histone deacetylation correlates with the transcriptional repression and silencing of genes. Several transcription factors, such as Mad/Max and YY1, bind histone deacetylases which stabilize nucleosomal structure and repress transcription (5, 6). Between these two processes there is a dynamic equilibrium which might be disturbed, for instance, in some atrophic and degenerative diseases.

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An important question is how might these multiprotein complexes be regulated, especially those repressor complexes containing histone deacetylases. Recent studies have shown that the key component in these complexes is the mSin3 protein which act as an adaptor molecule binding both transcription factors and histone deacetylases (1-4). mSin3 proteins also interact with several other co-repressors, such as SMRT, N-CoR, SAP18 and SAP30 (4, 7, 8). Interestingly, mSin3A protein also binds to MeCP2 protein which is known to bind to methylated CpG residues (9, 10). mSin3A recruits histone deacetylases to this complex and induces stable repression of genes (*see* 10).

There are two cloned mammalian mSin3 proteins, mSin3A and mSin3B (11, 12). Mammalian, as well as yeast Sin3, proteins contain paired amphipathic helices (PAH) which mediate protein-protein interactions (11). mSin3A is widely expressed in mammalian tissues and shows normally "doublet" proteins which is due to either translational initiation or alternative splicing (11, 12).

The purpose of this study was to find out whether the expression of mSin3 proteins is affected during neuronal apoptosis. Here we show that the expression of mSin3A but not mSin3B protein is highly upregulated during apoptosis induced both by serum withdrawal or treatments with etoposide, okadaic acid or trichostatin A in neuroblastoma 2a cells.

METHODS

Cell culture and induction of apoptosis. Mouse neuroblastoma 2a (Neuro 2a) cells (CCL 131) were obtained from American Type Culture Collection and cultured in Dulbecco's DMEM medium supplemented with 10% fetal calf serum (Gibco). Apoptosis was induced in exponentially growing cells. We have recently described in detail the models for neuronal apoptosis in Neuro 2a cells using serum withdrawal and etoposide (13) as well as histone deacetylase inhibitor trichostatin A (14) as apoptotic inducers. The final concentrations of apoptotic inducers used were 40 nM for etoposide (Sigma), okadaic acid (Calbiochem) and staurosporine (Sigma), and 2 μM for trichostatin A (Sigma).

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Western blot assays. Soluble nuclear protein samples were used in Western blot assays. Our preliminary experiment showed that mSin3A and mSin3B proteins were located in soluble nuclear protein fraction (see Figure 1). Proteins were isolated similarly to those for transcription factors, using the Dignam method (15) with slight modifications as described earlier in detail (16). Protein samples (10 μg) were solubilized in Laemmli-PAGE sample buffer, boiled for 5 min, resolved at 200 V on 6% SDS-PAGE gels and electrophoretically transferred to ECL-nitrocellulose membrane (Amersham). Membranes were blocked for 1 h at room temperature in PBS (phosphate-buffered saline) containing 3% BSA and 0.05% Tween-20. Membranes were probed for 2 h at room temperature with anti-mSin3A (Santa Cruz, AK-11, 200 μg/0.1 ml, dilution 1:2000) and mSin 3B (Santa Cruz, A-20, 200 μg/0.1 ml, dilution 1:1000). After washing, membranes were incubated with horse radish peroxidase -conjugated donkey anti-rabbit immunoglobulin $F(ab)^2$ fragment (Amersham) (dilution 1:4000) for 1 h. Results were detected by enhanced chemiluminescence kit (ECL-kit, Pierce) according to the manufacturer's manual.

Immunoprecipitation assays. Subconfluent Neuro 2a cells were labelled with ^{35}S-methionine (Amersham) (50 μCi/ml) for 12 h with or without apoptotic inducers in DMEM (without methionine) with 10% dialyzed FBS. After washing, cells were lysed in RIPA buffer (17) and after centrifugation (12 000 g) immunoprecipitated with rabbit polyclonal antibody against mSin3A (AK-11). Precipitation with protein A-Sepharose and SDS-PAGE were performed as described earlier (17).

Caspase-3 activity assays. Cytoplasmic extracts were prepared by the treatment of cells with hypotonic buffer (16). The activities of caspase-3 were assayed using fluorogenic substrate Ac-DEVD-AMC (Pharmingen). The substrate was used at a final concentration of 20 μM. Assays were performed according to the protocol of Pharmingen.

RESULTS

mSin3A and mSin3B are nuclear proteins. Figure 1 shows that mSin3A and mSin3B proteins are localized in nuclear fraction in neuroblastoma 2a cells. Cytoplasmic fraction did not show any mSin3A or mSin3B staining in Western blot assays. mSin3A protein appeared in "doublet", as observed in several other cell types (12, 18). The upper band shows the longer mSin3A isoform and lower the shorter mSin3A protein

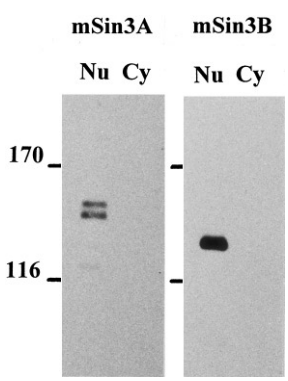

FIG. 1. Nuclear localization of mSin3A and mSin3B proteins in Neuro 2a cells. Nuclear (Nu) and cytoplasmic(Cy) proteins were isolated as described in METHODS. Ten μg of protein were used for SDS-PAGE and immunoblotting. mSin3A shows the "doublet" bands around 150 kD and mSin3B a single band of approx. 130 kD.

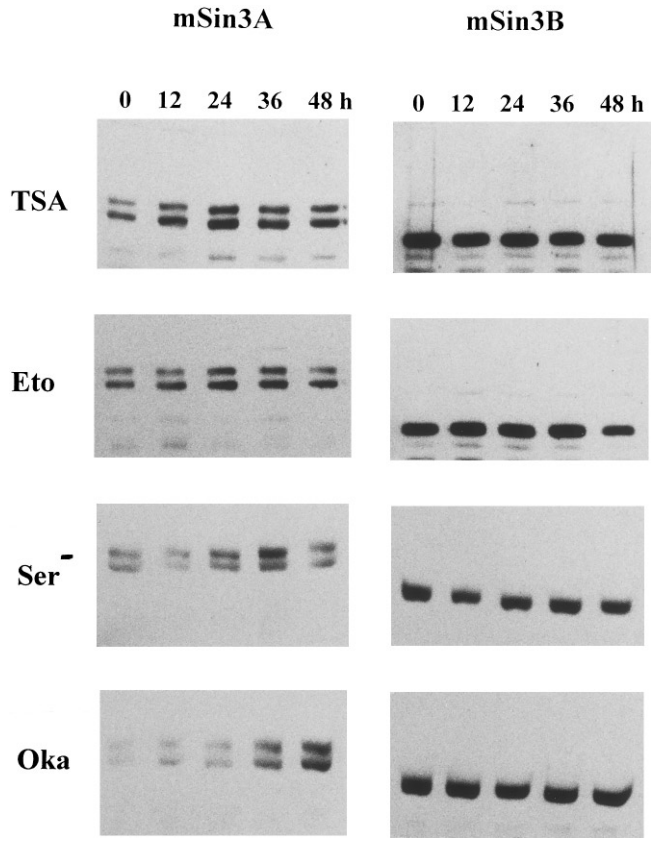

FIG. 2. Expression of mSin3A and mSin3B proteins after exposure to apoptotic inducers in Neuro 2a cells. Inducers used were: TSA (trichostatin A, 2 μM final conc.), Eto (etoposide, 40 nM final conc.), Ser$^-$ (serum withdrawal) and Oka (okadaic acid, 40 nM final conc.). Exposure times were 0 h, 12 h, 24 h, 36 h and 48 h.

(11). The molecular weight of mSin3A is around 150 kD and mSin3B approximately 130 kD, a little larger than predicted from the sequence (11).

Induction of mSin3A but not mSin3B protein expression during neuronal apoptosis. We have recently established and described the apoptosis models in Neuro 2a cells using serum withdrawal and treatments with etoposide, trichostatin A or okadaic acid as inducers (13, 14). Figure 2 shows that all these treatments induced a prominent increase in mSin3A protein level. The induction of mSin3A expression was selective since the protein level of mSin3B was unaffected by the apoptotic treatments. Interestingly, trichostatin A, an inhibitor of histone deacetylases (19), induced the most prominent upregulation of mSin3A proteins (Figure 2A). Increased protein levels appeared already 12 h after the exposure to trichostatin A. Both the upper and lower protein bands were nearly equally upregulated (Figure 2A). mSin3B shows a strong expression but does not show upregulation after use of any inducer (Figure 2B).

Induction is due to increased synthesis of mSin3A proteins. We also studied the effects of neuronal apoptosis inducers on mSin3A expression using metabolic

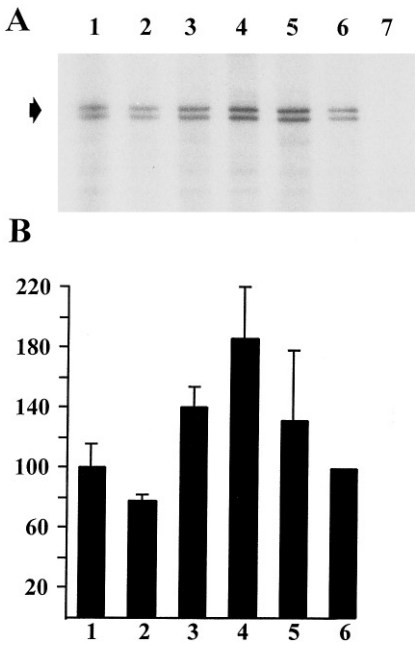

FIG. 3. Synthesis rate of mSin3A proteins during 12 h after the exposure to apoptotic inducers. **A.** A representative immunoprecipitation autoradiography. **B.** PhosphorImager (Storm, Molecular Dynamics) data from two separate experiments showing average pixel values (±S.D.) normalized to control value (100%). Apoptotic inducers used (lanes in A and columns in B); 1 (control sample), 2 (serum withdrawal), 3 (okadaic acid, 40 nM final conc.), 4 (trichostatin A, 2 μM final conc.), 5 (etoposide, 40 nM final conc.), 6 (staurosporine, 40 nM final conc.), 7 (precipitation without sample protein). Equal amount of radioactivity (10^7 cpm) was used for the precipitation of each sample.

labeling with ^{35}S-methionine and immunoprecipitation technique. Figure 3 shows that the synthesis rate of mSin3A proteins is significantly increased during 12 h labelling time after trichostatin A and etoposide treatments. Serum withdrawal, however, did not show any increase in the synthesis of mSin3A proteins for the first 12 h. This is in agreement with Western blot results which showed a late increase in mSin3A protein level during serum deprivation (Figure 2A).

Upregulation of mSin3A protein expression precedes caspase-3 activation during neuronal apoptosis. Our studies on apoptosis in Neuro 2a cells (13, 14), as well as other reports in literature (20, 21), have shown that the activation of caspase-3 is a reliable quantitative parameter for neuronal apoptosis. Figure 4 shows that all apoptotic inducers increased the activity of caspase-3 in cytoplasm. The highest activities were recorded after 48 h, except after etoposide treatment when the highest level was reached after 36 h. Interestingly, the appearance of the highest level of mSin3A preceded the activation of caspase-3 and the execution phase of neuronal apoptosis (Figures 2 and 4). For instance, the level of mSin3A proteins was the highest after 24 h exposure to trichostatin A but caspase-3 activities reached the highest level after 48h.

DISCUSSION

Degenerative diseases, such as Alzheimer's disease, are the most devastating neuronal diseases. Degeneration may appear as a neuronal apoptosis-necrosis continuum (22) or as neuronal atrophy, appearing *e.g.* during aging (23). Molecular mechanisms underlying degenerative processes in neurons are still largely unknown. However, outstanding progress has been made in the studies on genetic regulation of neuronal apoptosis (see *e.g.* 24). We have been interested in the role of genetic repression in neuronal degeneration. We have observed, for instance, that neuronal degeneration induced by L-glutamate involves a dramatic change in YY1 complexes both in hippocampal and cerebellar granule neurons (25). YY1 is a well-characterized repressor protein which binds, for example, histone deacetylase 2 (HDAC2) (6).

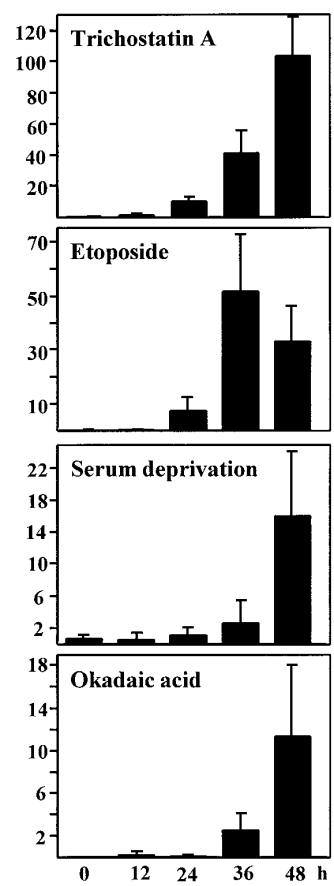

FIG. 4. Caspase-3 activation in different apoptosis models. Exposure times: 0 h, 12 h, 24 h, 36 h and 48 h. Values of caspase-3 activity are expressed as nmol AMC (7-amino-4-methyl coumarin) per second and gram of cytoplasmic protein. Values are means ±S.D. (n=3). Exposure times of 36 h and 48 h show the statistically significant ($p < 0.01$) increases in caspase-3 activity.

Regulation of dynamic equilibrium between histone acetylation and deacetylation provides an interesting mechanism either to induce or enhance atrophic and degenerative processes in neuronal cells. Reduced acetylation or enhanced deacetylation of histones will induce transcriptional repression or silencing of targeted genes (1-4). mSin3 repressor proteins are adaptor proteins which recruit a repressive complex with histone deacetylases to induce the repression of transcription (4, 7, 8). Interestingly, we observed a significant upregulation in the synthesis and expression level of mSin3A protein during apoptosis in neuroblastoma cells. The expression of mSin3B protein was not affected which suggests a selective regulation of mSin3 proteins, and it seems that mSin3A proteins are inducible in stresses which induce apoptosis.

Trichostatin A induced the strongest induction of mSin3A protein expression as well as the activation of caspase-3 as compared to other apoptotic inducers. Synthesis rate and the protein level of mSin3A were significantly upregulated already after 12 h period. Trichostatin A, a microbial metabolite, is a potent reversible inhibitor of mammalian histone deacetylases (19). Several studies have shown that the inhibition of histone deacetylases with trichostatin A induces the hyperacetylation of histones and regulates the transcriptional activity of several genes (19, 26). We have recently reported that micromolar concentrations of trichostatin A are effective apoptotic inducers both in Neuro 2A cells and cerebellar granule cells (14). Interestingly, the inhibitor of histone deacetylases induced a strong expression of mSin3A proteins which suggests a feedback regulation. However, this response was not specific for trichostatin A treatment but rather was related to apoptosis since it appeared also in apoptosis induced by other inducers.

mSin3A is a key component, for instance, in the formation a complex between DNA-binding Mad/Max factors and histone deacetylases (5). c-Myc proto-oncogene and its binding partners Mad/Max are known to be involved in the induction of apoptosis (*see* 27). For instance, c-Myc may contribute to the delayed neuronal apoptosis in hippocampus following transient forebrain ischemia (28). At present, however, it is not known what are the transcription factors which bind mSin3 proteins and mediate transcriptional repression.

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