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Immediate Early Gene IEX-1 Induces Astrocytic Differentiation of U87-MG Human Glioma Cells

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Abstract The immediate early response gene IEX-1 is involved in the regulation of apoptosis and cell growth. In order to increase the apoptotic sensitivity to chemotherapeutic drugs and γ-ray, we attempted to establish U87-MG human glioma cell line expressing IEX-1. Unexpectedly, however, transfection of IEX-1 into U87-MG glioma cells resulted in morphological changes to astrocytic phenotype and increase in glial differentiation marker proteins, S-100 and glial fibrillary acidic protein (GFAP). Glial cell differentiation was used to examine in rat C6 glioma cell line, since this cell line express astrocytic phenotypes by increase in intracellular cAMP concentration. Stimulation of human U87-MG glioma cells by membrane-permeable dibutyryl cAMP (dbcAMP) not only elicited their morphological changes but also induced expression of IEX-1 as well as S-100 and GFAP. H89, an inhibitor of protein kinase A (PKA), blocked dbcAMP-induced morphological changes of U87-MG cells and expression of IEX-1. In contrast, morphological changes and expression of S-100 and GFAP induced by IEX-1 were not affected by H89. Morphological changes induced by dbcAMP were totally abolished by functional disruption of IEX-1 expression by anti-sense RNA. These results indicate that IEX-1 plays an important role in astrocytic differentiation of human glioma cells and that IEX-1 functions at downstream of PKA. J. Cell. Biochem. 100: 256–265, 2007.

Key words: IEX-1; glioma; differentiation; cAMP; signal transduction

The human immediate early gene X-1 (IEX-1, also known as Dif1 and PRG1), the human ortholog of murine gly96 [Charles et al., 1993], was initially identified in human squamous carcinoma cells as a X-radiation-inducible immediate early gene [Kondratyev et al., 1996]. The IEX-1 gene encodes a protein of 156-amino acids and shares no significant sequence similarities with any other known protein. The protein undergoes post-translational glycosylation to yield a product of 27–29 kDa. This gene is also induced by stress stimuli, ultraviolet B radiation [Kumar et al., 1998] and ceramide [Pietzsch et al., 1997], vitamin D_3 [Kobayashi et al., 1998], and growth factors such as epidermal growth factor [Kumar et al., 1998; Schäfer et al., 1996, 1999]. Recent studies indicate that IEX-1 is implicated in apoptotic signaling and cell cycle control [Reviewed by Kumar et al., 2003; Wu, 2003]. We have investigated the mechanism of hepatocyte apoptosis in order to gain the basis for the development of new therapeutic approach to human fluminant hepatitis [Nagaki et al., 2000; Osawa et al., 2001a,b]. We have identified IEX-1 gene as a tumor necrosis factor-α (TNF-α)-induced and nuclear factor-κB (NF-κB)-dependent gene during hepatocyte apoptosis using cDNA microarray [Osawa et al., 2003]. In Hc human hepatocytes, IEX-1 appeared to enhance TNF-α-induced apoptosis by blockage of Akt survival signal.

Malignant gliomas are the most common malignant brain tumors and are usually incurable. The infiltrative growth patter of these tumors precludes curative neurosurgery and tumor cells are usually resistant to irradiation, chemotherapy, or immunotherapy. Therefore, differentiation-inducing therapy, which is the most successful for the treatment of acute

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myelocytic leukemia and is promising in certain osteosarcoma, has recently proposed to be a novel potential approach to treat malignant gliomas [Dai and Holland, 2003]. In order to gain insight into the approach to treatment of malignant gliomas, we have examined the apoptotic signal pathways in cultured human glioma cells treated with chemotherapeutic agents and γ-ray [Sawada et al., 2000, 2001, 2004; Noda et al., 2001; Hara et al., 2004]. In the present study, we have attempted to test the possibility that IEX-1 might enhance apoptosis of human glioma cells induced by therapeutic treatments, such as chemotherapeutic agents and γ-ray. Unexpectedly, however, we found that U87-MG human glioma cells overexpressing IEX-1 underwent morphological changes to astrocytic phenotype and increase in glial differentiation marker proteins, S-100 [Tabuchi et al., 1981] and glial fibrillary acidic protein (GFAP) [Takanaga et al., 2004]. Elevated cAMP and the consequent activation of protein kinase A (PKA) appeared to differentiate U87-MG glioma cells to express astrocytic phenotype, as previously observed in rat C6 glioma cell line [Willingham, 1976; Yoshimura et al., 1997; Takanaga et al., 2004], which is often used as a model system of glial cell differentiation [Harris et al., 1980]. During differentiation provoked by membrane-permeable dibutyryl cAMP (dbcAMP), expression of IEX-1 was induced. When IEX-1 expression was suppressed by anti-sense RNA, dbcAMP was unable to stimulate differentiation of U87-MG glioma cells. These results suggest that IEX-1 is deeply involved in differentiation of human glioma cells. As far as we know, this is the first report that links to IEX-1 to cellular differentiation process.

MATERIALS AND METHODS

Materials

U87-MG human glioma cells were obtained from Human Science Research Resource Bank (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM), dbcAMP, and H89 were from Sigma (St. Louis, MO). Fetal bovine serum (FBS) and penicillin/streptomycin were from ICN (Aurora, OH). pIRES1neo and pIRES2-EGFP plasmid vectors were from Clontech Laboratories (Palo Alto, CA). Trans-IT polyamine transfection reagent was from Pan Vera (Madison, WI). Antibodies against IEX-1S/L (N-17), S-100, and GFAP, and anti-goat IgG HRP-coupled secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-actin antibody was from Calbiochem (La Jolla, CA). Anti-mouse IgG HRP-coupled secondary antibody and ECL Western blot detection system were from Amersham Biosciences (Buckinghamshire, UK). All other reagents used were of the highest analytical grade available.

Cell Culture

U87-MG human glioma cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C [Hara et al., 2004]. For differentiation, 5×10^4 cells were plated on 100-mm dishes and cultured for 24 h. The cells were then rinsed twice with PBS and incubated for 1 h in DMEM supplemented with 1% FBS. If necessary, 50 μM H89 was included in the medium. Differentiation was initiated by the addition of 1 mM dbcAMP. Control cultures were carried out in DMEM supplemented with 1% FBS only.

Expression of IEX-1 and Anti-IEX-1

The full-length human IEX-1 cDNA was obtained as described previously [Osawa et al., 2003] and subcloned into pIRES1neo or pIRES2-EGFP plasmid vector by digestion with *Eco*R1. The former vector was used for stable transfection and the latter was for transient transfection. Increased IEX-1 protein expression was observed in the cells transfected with the plasmids containing IEX-1 cDNA in the sense direction. In contrast, IEX-1 protein expression was canceled in the cells with the plasmids containing IEX-1 cDNA in the antisense direction. Therefore, the latter plasmids were designated plasmids with anti-IEX-1. The expression plasmids were transfected into U87-MG cells using Trans-IT according to the manufacturer's instructions. Stable transfectants were selected in DMEM supplemented with 10% FBS containing 0.5 mg/ml G418.

Western Blot Analysis

Total cellular protein extracts were used for the Western blot analysis. The cells were solubilized in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM EGTA, 1%

Triton-X, 0.1% SDS, 1% deoxycholic acid, 0.3 mM PMSF, 30 μg/ml (L-3-trans-carboxyoxirane-2-carboryl)-L-leucylagmatine (E64), 1 mM sodium orthovanadate, 10 mM sodium fluoride, 0.1 mM sodium molybdate, 0.5 mM 4-deoxypyridoxine). The proteins were separated by SDS–PAGE, and were electrophoretically transferred onto polyvinylidene difluoride membranes. The membranes were first incubated with the indicated primary antibody, and then incubated with the anti-mouse or anti-goat IgG horseradish peroxidase-coupled secondary antibody. Detection was performed with an ECL system.

Cytoprocess Formation

More than 200 cells were counted in at least three independent experiments, and cytoprocess formation was evaluated as the percentage of cells with processes (with process length more than fourfold the diameter of the cell body) relative to the total number of cells. All errors were calculated as SD.

RESULTS

IEX-1 Overexpression Leads to Astrocytic Differentiation of Human Glioma Cells

In the previous study, we have disclosed that IEX-1 enhanced TNF-α-induced apoptosis in human Hc cells [Osawa et al., 2003]. The rate of apoptosis is enhanced by IEX-1 in several types of cells [Arlt et al., 2001, 2003; Schilling et al., 2001]. Therefore, we thought that IEX-1 might enhance apoptotic death of incurable human glioma cells in response to therapeutic treatments, such as chemotherapeutic drugs and γ-ray. To test this possibility, we tried to establish U87-MG human glioma cells overexpressing IEX-1 protein by transfection of pIRES1neo plasmid vector containing IEX-1 cDNA. During selection with G418, unexpectedly, we found that the surviving cells changed their shape from flat polygonal appearance to spindle-shape with processes. It is reasonable to speculate that these morphological changes were caused by overexpressed IEX-1. However, the effects of G418 and prolonged culture could not be excluded. Therefore, the effect of IEX-1 was analyzed in transient transfection system. For this purpose, IEX-1 cDNA was subcloned into pIRES2-EGFP plasmid vector. U87-MG cells were transiently transfected with pIRES2-EGFP plasmid vector without or with IEX-1 cDNA and shape of GFP-positive cells were observed. In vector-transfected cells, the shapes of transfected cells with green GFP fluorescence were polygonal and were almost same as those without GFP fluorescence (data not shown). However, cells transiently overexpressing IEX-1 protein with green GFP fluorescence underwent drastic morphological changes, characterized by cell rounding and formation of long processes (Fig. 1). The level of IEX-1 protein in vector-transfected cells was below the detectable level by Western blotting. In these cells S-100 protein, a glial differentiation marker [Tabuchi et al., 1981], was very low. However, S-100 protein increased drastically in cells overexpressing IEX-1.

In order to further examine the role of IEX-1 in glial cells, U87-MG clones stably overexpressing IEX-1 protein were generated and several independently isolated clones analyzed for IEX-1 expression by Western blot. All clones demonstrated elevated IEX-1 expression compared with vector-transfected control lines and their morphological appearance were different from those of control lines (Fig. 2). Cells expressing relatively low level of IEX-1 became spindle-shaped with processes. Cells expressing high level of IEX-1 were no longer flat but rather rounded with longer processes, characteristic of cells exhibiting astrocytic phenotypes. The levels of S-100 protein and GFAP, glial differ-

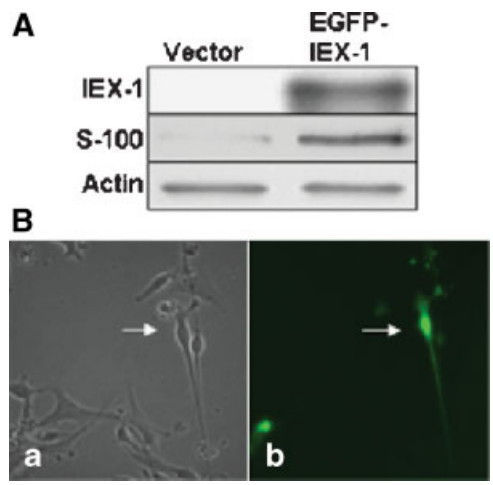

Fig. 1. Changes in U87-MG cells transiently transfected with IEX-1 cDNA. U87-MG cells were transiently transfected with pIRES2-EGFP plasmid vector containing IEX-1 cDNA (EGFP-IEX-1) for 24 h. **A**: The extracted proteins were subjected to Western blot analysis with anti-IEX-1, S-100, and actin antibodies. **B**: Cell morphology was analyzed by a phase contrast microscope (**a**) and GFP fluorescence of the same scene was by a fluorescent microscope (**b**). The results shown are representatives from at least three experiments.

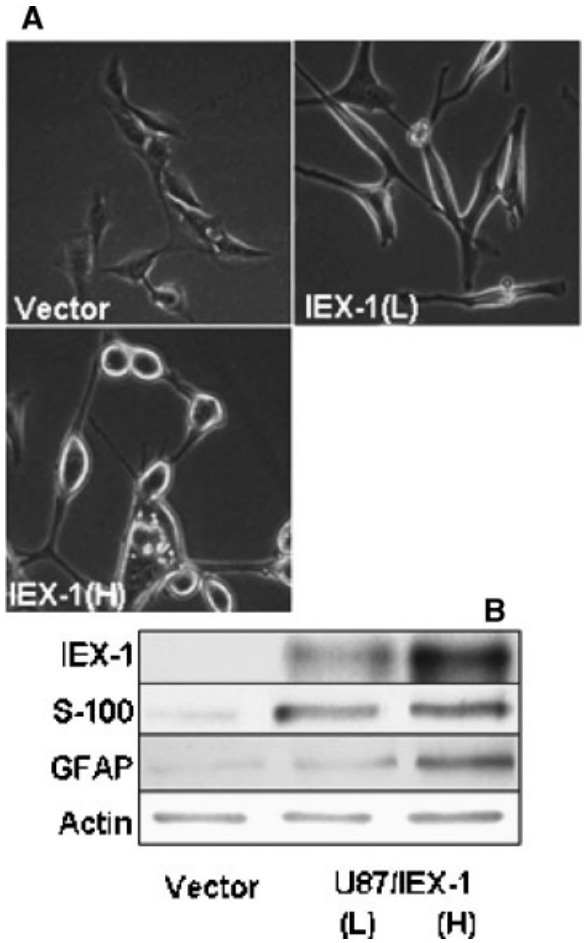

Fig. 2. Constitutive overexpression of IEX-1 affected cell morphology and expression of S-100 and GFAP. U87-MG cells were transfected with pIRES1neo vector containing IEX-1 cDNA. After selection with G418 (0.5 mg/ml), the colonies expressing two different levels (H, high and L, low) of IEX-1 were obtained. **A**: Phase-contrast micrographs of cells. **B**: The extracted proteins were subjected to Western blot analysis with anti-IEX-1, S-100, GFAP, and actin antibodies. The results shown are representatives from at least three experiments.

entiation markers [Tabuchi et al., 1981; Takanaga et al., 2004], increased directly proportional to that of IEX-1. From these results, we speculated that IEX-1 might be implicated in differentiation of glioma cells.

IEX-1 Expression is Induced During Astrocytic Differentiation Induced by dbcAMP

It is well-known that differentiation (morphological changes to astrocytic phenotype and increases of glial differentiation maker proteins) of rat C6 glioma cells occurs under conditions that elevate intracellular cAMP concentration [Willingham, 1976; Yoshimura et al., 1997; Takanaga et al., 2004]. Therefore, human U87-MG glioma cells were treated with membrane-permeable dbcAMP (1 mM). Cell shapes were gradually changed (Fig. 3). The number of cells with processes, the length of which were more than fourfold the diameter of the cell body, increased time dependently for up to 72 h. At 24 h cells extended processes and at 48 h cells became spindle-shape with long processes. During morphological changes, the levels of S-100 protein and GFAP, glial differentiation markers [Tabuchi et al., 1981; Takanaga et al., 2004], increased (Fig. 4). These results indicate that differentiation of U87-MG glioma cells was induced by the increase in intracellular cAMP concentration as observed in rat C6 cells [Willingham, 1976; Yoshimura et al., 1997; Takanaga et al., 2004]. These morphological changes and expression of S-100 protein and GFAP induced by dbcAMP were inhibited by H89, an inhibitor of PKA, indicating that the effect of cAMP was mediated by PKA. During dbcAMP-induced differentiation, expression of IEX-1 protein was induced. When cells were preincubated with H89, dbcAMP-induced IEX-1 expression was almost abolished. In contrast, morphological changes induced by IEX-1 was not affected by H89 (Fig. 5). These results suggest the possibility that IEX-1 functions at downstream of PKA.

Functional Disruption of IEX-1 by Anti-Sense RNA Blocked dbcAMP-Induced Morphological Changes

To further investigate the role of IEX-1, we tried to functionally inactive it. We found that IEX-1 expression by pIERS1neo vector was considerably suppressed by the pIRES-EGFP plasmid containing IEX-1 cDNA in the anti-sense direction (Fig. 6), most probably due to the production of anti-sense RNA. Therefore, this anti-IEX-1 plasmid was used for inactivation of IEX-1. The increased expression of S-100 protein in U87-MG cells stably transfected with IEX-1 was suppressed by anti-IEX-1. Most notably, the cell shape was drastically changed. The cells stably overexpressing IEX-1 extended long processes. When stably overexpressing IEX-1 was canceled by transient transfection with pIRES-EGFP plasmid containing anti-IEX-1, the cells, which were identified by green GFP fluorescence, lost long processes and became rounded. In cells expression of IEX-1

was functionally disrupted by anti-IEX-1, dbcAMP no longer induced morphological changes to astrocytic phenotype (Fig. 7).

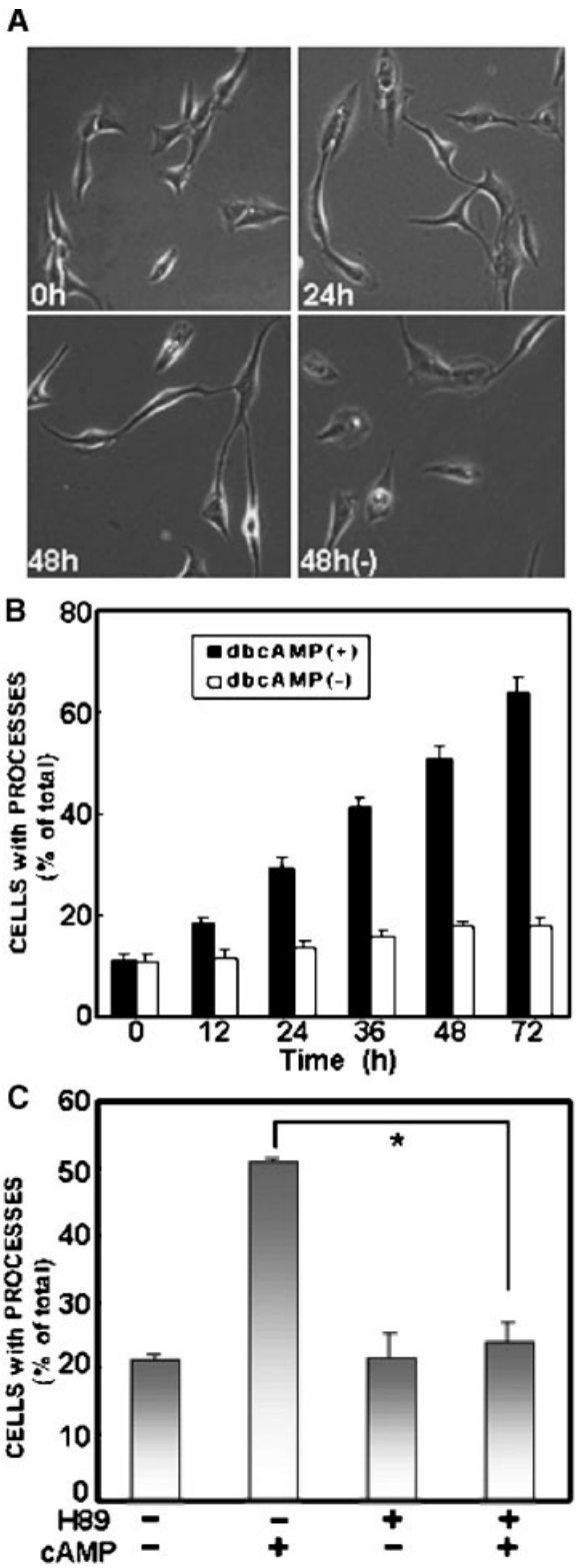

Fig. 3. Morphological changes of U87-MG cells induced by dbcAMP. U87-MG cells were treated with 1 mM dbcAMP for the indicated periods. **A**: Phase-contrast micrographs of the cells. **B**: Quantification of the process formation. Cells with processes longer than four diameters of cell body were counted among at least 200 cells. The data represent averages of three independent experiments. **C**: Cells were treated with 1 mM dbcAMP in the presence or absence of 50 μM H89 for 48 h. Cells with processes longer than four diameters of cell body were counted among at least 200 cells. The data represent averages of three independent experiments.

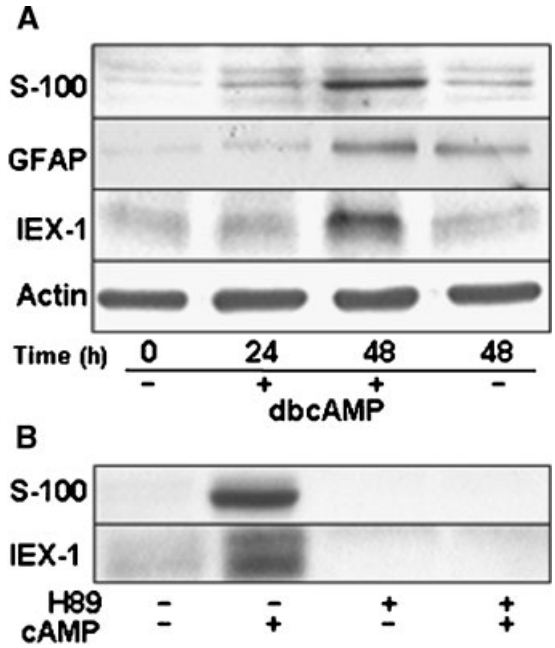

Fig. 4. Expression of S-100 and GFAP proteins induced by dbcAMP. **A**: U87-MG cells were treated with 1 mM dbcAMP for the indicated periods. **B**: U87-MG cells were incubated with 1 mM dbcAMP in the presence or absence of 50 μM H89 for 48 h. The extracted proteins were subjected to Western blot analysis with anti-IEX-1, S-100, GFAP, and actin antibodies. The results shown are representatives from at least three experiments.

DISCUSSION

IEX-1 is a growth- and stress-associated early response gene implicated in the regulation of cell cycle progression and proliferation [Kobayashi et al., 1998; Schäfer et al., 1999; Grobe et al., 2001] as well as apoptosis [Wu et al., 1998; Arlt et al., 2001; Schilling et al., 2001; Osawa et al., 2003]. However, IEX-1 appears to exert apparently contradictory effects, depending on the type of cells, stimuli and its expressed forms. In some types of cells IEX-1 has been shown to inhibit apoptosis [Wu et al., 1998; Domachowske et al., 2000; Zhang et al., 2002]. On the other hand, IEX-1

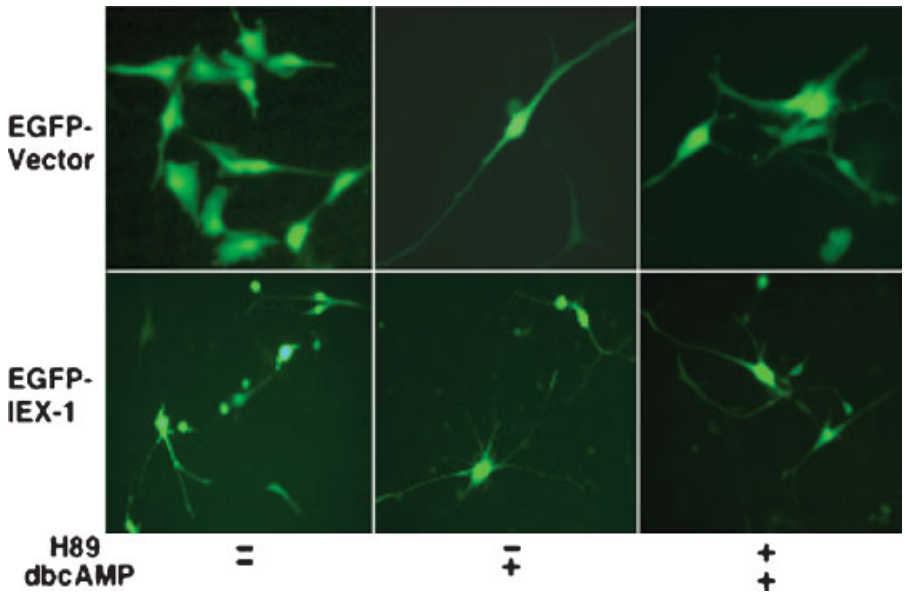

Fig. 5. IEX-1-induced morphological changes were minimally affected by dbcAMP or H89. U87-MG cells transiently transfected with pIRES2-EGFP vector alone or pIRES2-EGFP plasmid containing IEX-1 cDNA were treated with 1 mM dbcAMP in the presence or absence of 50 μM H89 for 48 h. GFP fluorescence was analyzed by a fluorescent microscope. The results shown are representatives from at least three experiments.

accelerates apoptosis in keratinocytes [Schilling et al., 2001] and epithelial tumor cells [Arlt et al., 2001]. There are two alternatively spliced forms of IEX-1. The longer IEX-1 transcript with 37-amino acid insertion, called IEX-1L, is regarded as an apoptosis inhibitor in NF-κB-mediated cell survival [Wu et al., 1998; Domachowske et al., 2000], whereas IEX-1S, a shorter original IEX-1 transcript promotes apoptosis [Arlt et al., 2001; Grobe et al., 2001; Schilling et al., 2001]. Interestingly, it has been proposed by two independent investigators that IEX-1S may promote cell proliferation under favorable growth condition but facilitate apoptosis under unfavorable conditions [Arlt et al., 2001; Schilling et al., 2001]. More recently, IEX-1 has been shown to regulate stretch-induced hypertrophy of cardiomyocytes [De Keulenaer et al., 2002] and vascular smooth muscle cells [Schulze et al., 2003]. Therefore, IEX-1 obviously generates cellular signals implicated in proliferation, death, and survival signaling pathways. However, the exact molecular mechanisms of IEX-1-mediated cellular response are not fully disclosed yet.

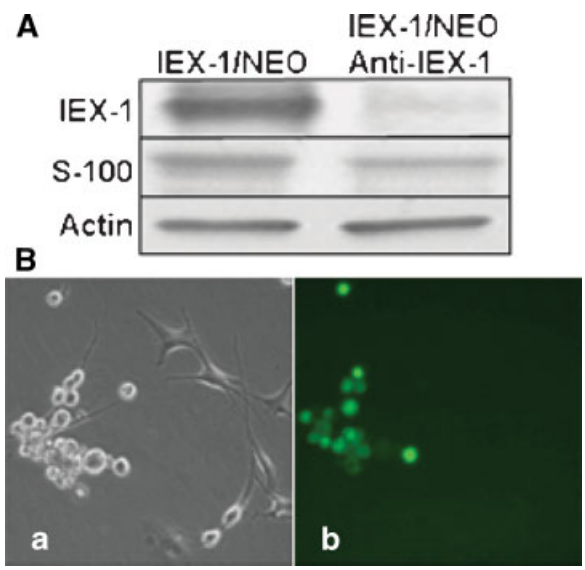

Fig. 6. Effects of anti-IEX-1 on protein expression and cell morphology in U87-MG cells stably transfected with IEX-1. U87-MG cells stably transfected with pIRES1neo vector containing IEX-1 cDNA were transiently transfected with pIRES2-EGFP vector containing anti-IEX-1 for 48 h. **A**: The extracted proteins were subjected to Western blot analysis with anti-IEX-1, S-100, and actin antibodies. **B**: Cell morphology was analyzed by a phase contrast microscope (**a**) and GFP fluorescence of the same scene was by a fluorescent microscope (**b**). The results shown are representatives from at least three experiments.

Recent studies have characterized functional interaction of IEX-1 with other functional molecules. IEX-1 potentiated activation of extracellular signal-regulated kinase (ERK) in response to various growth factors and was phosphorylated by ERK [Garcia et al., 2002]. IEX-1 attenuated NF-κB activation in 293 cells, although it was a NF-κB-dependent gene [Arlt et al., 2003]. In Hc hepatocytes, IEX-1 inhibited Akt activation and subsequent expression of

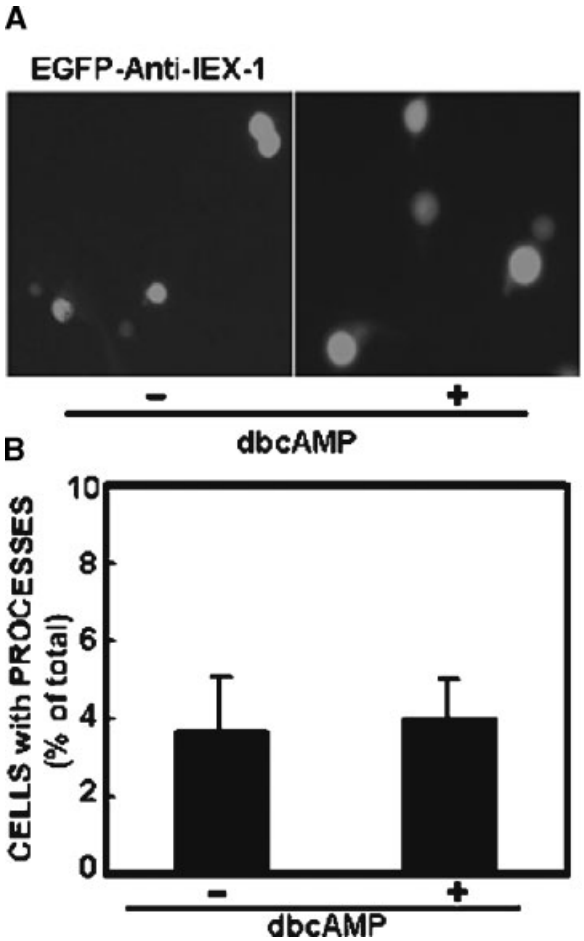

Fig. 7. Inhibition of dbcAMP-induced morphological changes by anti-IEX-1. U87-MG cells were transiently transfected with pIRES2-EGFP vector containing anti-IEX-1 in the presence or absence of 1 mM dbcAMP for 48 h. **A**: GFP fluorescence was analyzed by a fluorescent microscope. The results shown are representatives from at least three experiments. **B**: Quantification of the process formation. At least 100 cells with GFP fluorescence were counted in three separate experiments. The results were presented as the percentage of cells with processes relative to the total number of GFP-positive cells.

Mcl-1, a member of anti-apoptotic Bcl-2 family [Osawa et al., 2003]. Moreover, several proteins that modulate apoptosis have been identified by yeast two-hybrid screening using IEX-1 as a bait [Kumar et al., 2004].

There is little information regarding the role of IEX-1 in the nervous system. Our present study demonstrates, as far as we know for the first time, the possible role of IEX-1 in the cells of neuronal origin. The expression of IEX-1 was induced during astrocytic differentiation of U87-MG human glioma cells induced by an intracellular cAMP-elevating agent, dbcAMP in PKA-dependent fashion. Overexpression of IEX-1 in human glioma cells resulted in their morphological changes to astrocytic phenotype and increase in glial differentiation marker proteins, S-100 and GFAP. On the other hand, dbcAMP failed to stimulate differentiation of U87-MG glioma cells, if anti-IEX-1 anti-sense RNA was expressed. These results suggest that IEX-1 is deeply involved in differentiation of human glioma cells. IEX-1 could be a potential candidate molecule for differentiation-inducing therapy [Dai and Holland, 2003] of human gliomas. The present findings provided several important issues to be resolved in the future study for understanding the role of IEX-1 in cellular responses. For example, (1) modulation of IEX-1 expression by cAMP/PKA system, (2) control mechanism of S-100 and GFAP expression by IEX-1, (3) regulation of cytoskeletal organization and focal adhesion by IEX-1, and (4) the role of IEX-1 in other cells (neurons, normal glial cells, etc.) from the neuronal origin.

IEX-1 transcription is controlled by a variety of factors, such as irradiation, growth factors, cytokines, TNF-α, cytokines, etc. In accordance with this, the promoter region of IEX-1 gene contains multiple consensus sequences for a large number of transcription factors [Pietzsch et al., 1998; Schäfer et al., 1998a; Im et al., 2002a,b]. Among them, NF-κB [Pietzsch et al., 1998; Schäfer et al., 1998b; Huang et al., 2002], sp1 [Schäfer et al., 1998a; Im et al., 2002b], c-myc [Huang et al., 2002], and p53 [Huang et al., 2002; Im et al., 2002b] are shown to play major roles. PKA/CREB (cAMP-responsive element-binding protein) pathway [Hai and Hartman, 2001] could be involved in cAMP-mediated glial cell differentiation [Dai and Holland, 2003]. Although IEX-1 expression was induced by dbcAMP in human glioma U87-MG cells, CRE (cAMP-responsive element) was not identified in the promoter region of IEX-1 [Pietzsch et al., 1998; Schäfer et al., 1998a; Im et al., 2002a,b]. It was shown recently that cAMP-induced GFAP expression in C6 glioma cells was mediated by delayed STAT3 activation via autocrine interleukin-6 (IL-6) [Takanaga et al., 2004]. Therefore, transcription factor(s) in addition to CREB, which promotes transcription of IEX-1, should be activated during dbcAMP-induced differentiation of U87-MG glioma cells.

Most of "so-called" immediate early genes are induced in response to stress condition and function as transcription factors to regulate the

transcriptional activity of other genes. However, the solid evidence that IEX-1, which does not have typical DNA-binding domain [Kondratyev et al., 1996; Wu, 2003], functions to control transcriptional activity has not yet been presented. In the present study, the expression of at least two proteins, S-100 and GFAP were stimulated by overexpression of IEX-1. A recent detailed subcellular localization analysis [Kruse et al., 2005] has revealed that IEX-1 is recruited to and exported from the nucleus. This typical movement to and from nucleus is common for the molecules involved in the regulation of gene transcription or reorganization of nuclear proteins. Therefore, it is very likely that IEX-1 modulates various cellular responses through, at least in part, transcriptional control of other genes, although the control mechanism remains elusive.

Morphological changes of the cells are controlled by the reorganization of cytoskeleton and focal adhesions. The small GTP-binding proteins of the Rho subfamily, Rho, Rac, and Cdc42, are mediators in signal transduction pathways that regulate cytoskeletal organization [Hall, 1994; Takai et al., 1995]. Rho subfamily was shown to play a crucial role in determining the shape of astrocytes [Suidan et al., 1997]. In vascular smooth muscle cells, morphological changes, characterized by formation of long-branched processes, induced by increase in intracellular cAMP concentration is recently shown to be caused by inhibition of Rac function [Pelletier et al., 2005]. Apparently similar morphological changes of human glioma cells induced by dbcAMP could also be explained by the same mechanism. Rho subfamily GTP-binding proteins should be implicated in the regulation of cytoskeletal network and focal adhesions by expressed IEX-1, but the understanding of its exact molecular mechanism is a subject for future study.

In summary, we have shown in this study that IEX-1 is deeply involved in the process of differentiation of human glioma cells to express astrocytic phenotypes. The elucidation of the unanswered problems listed above will provide a clearer picture of IEX-1 function in the cells of neuronal origin and beneficial information on differentiation-inducing therapy. Further investigation is under current progress in our laboratory.

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