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Title: Regulation of glutathione synthesis *via* interaction between GTRAP3-18 and EAAC1 at plasma membrane

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Running title: GTRAP3-18 regulates glutathione synthesis

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Abbreviations used in this paper: DHK, dihydrokainate; EAAC1, excitatory amino acid carrier-1; EAAT, excitatory amino acid transporter; GSH, glutathione; GTRAP3-18, glutamate transport associated protein 3-18; LAβH, L-aspartate-β-hydroxamate; MeβCD, methyl-β-cyclodextrin; PMA, phorbol-12-myristate-13-acetate; TBOA, DL-threo-β-benzyloxyaspartate

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ABSTRACT

Regulation of the cysteine transporter EAAC1 for intracellular glutathione (GSH) content was investigated using HEK293 cells as a model system. GSH content was significantly reduced by L-aspartate-β-hydroxamate (50-250 μM), an inhibitor of both EAAC1 and GLT1, both of which are transporters to take up cysteine, while dihydrokainate (1-100 μM), a specific inhibitor of GLT1, failed to do so. This indicates that EAAC1 is involved in GSH content in HEK293 cells. We examined the effect of GTRAP3-18, which is capable of interacting with EAAC1. The GSH content decreased when the GTRAP3-18 protein level at the plasma membrane was increased by methyl-β-cyclodextrin (250 μM), rendering the cells more vulnerable to oxidative stress. Intracellular GSH increased when the GTRAP3-18 protein level at the plasma membrane was decreased by antisense oligonucleotides, rendering the cells more resistant to oxidative stress. Furthermore, we found that the increase in GSH content produced by stimulating protein kinase C, a translocator and activator of EAAC1, was inhibited by an increase in cell surface GTRAP3-18 protein. These results show GTRAP3-18 to negatively and dominantly regulate cellular GSH content *via* interaction with EAAC1 at the plasma membrane.

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Introduction

GSH helps maintain the sulfhydryl groups of proteins in the reduced state and the iron heme in the ferrous state. It also serves as a reducing agent for glutaredoxin, DNA, toxic peroxides, reactive oxygen species and free radicals. GSH is synthesized from glutamate, glycine and cysteine. As for intracellular cysteine availability for GSH synthesis, two different mechanisms exist. One mechanism, which is found in astrocytes and immature neurons, is uptake of cystine, which is then converted to cysteine (Cho and Bannai, 1990; Murphy et al., 1990). The other mechanism, which is found in mature neurons, being unable to take up cystine (Sagara et al., 1993), is direct uptake of cysteine (Sagara et al., 1993; Dringen, 2000). In cultured cells compared with tissues, cystine uptake might be downregulated (McBean, 2002). In fact, human embryonic kidney (HEK293) cells have markedly low ability to take up cystine (Shih and Murphy, 2001) compared with brain tissues (Pacchioni et al., 2007).

In mature neurons, which can not take up cystine, cysteine is the rate-limiting factor for GSH synthesis (Dringen et al., 1999; Dringen, 2000; Dringen and Hirrlinger, 2003). Cell culture studies suggest *e*xcitatory *a*mino *a*cid *c*arrier-1 (EAAC1) to be a neuronal cysteine transporter (Shanker et al., 2001; Chen and Swanson, 2003; Himi et al., 2003). Moreover, Aoyama et al. (Aoyama et al., 2006) demonstrated EAAC1 to be an essential transporter of cysteine needed for GSH synthesis, as evidenced by EAAC1 gene-deficient mice displaying both a low level of neuronal GSH and vulnerability to oxidative stresses. EAAC1 is a member of the family of sodium-dependent excitatory amino acid transporters (EAATs). EAAC1 is widely expressed in neurons in the mature brain (Rothstein et al., 1994), but its contribution to glutamate reuptake from the synaptic cleft is so minuscule (reviewed in Danbolt, 2001) that its major function may be cysteine transport. The structural requirements of EAAC1 for glutamate and cysteine transport appear to be different, since point mutations in the EAAC1 primary structure result in clear dissociation of the transport capability for each amino acid (Bendahan et

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al., 2000). GTRAP3-18 (*g*lutamate *tr*ansport *a*ssociated *p*rotein for EAAC1) is a membrane-associated protein that interacts with EAAC1 (Lin et al., 2001) and negatively modulates EAAC1-mediated glutamate reuptake *in vitro* as well as *in vivo* (Lin et al., 2001; Butchbach et al., 2002; Butchbach et al., 2003). However, since differential structural conformation of EAAC1 is required for cysteine and glutamate transport, it remains to be established whether GTRAP3-18 is capable of regulating cysteine uptake and intracellular GSH content.

In order to answer this question, we used HEK293 cells, which express only EAAC1 among EAATs (Lin et al., 2001) and have little ability to take up cystine. We demonstrated GTRAP3-18 to dominantly and negatively determine the intracellular GSH content.

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Materials and Methods

Materials. L-aspartate-β-hydroxamate (LAβH), methyl-β-cyclodextrin (MeβCD), 4β,9α,12β,13α,20-pentahydroxytiglia-1,6-dien-3-one 12-tetradecanoate 13-acetate (PMA), 4α,9α,12β,13α,20-pentahydroxytiglia-1,6-dien-3-one 12-tetradecanoate 13-acetate (4α-PMA), quisqualate and anti-actin antibody were purchased from Sigma-Aldrich (St. Louis, MO). DL-threo-β-benzyloxyaspartate (TBOA) and dihydrokainate (DHK) are from TOCRIS (Bristol, UK). Anti-EAAC1 antibody was obtained from Alpha Diagnostic International (San Antonio, TX) and anti-GTRAP3-18 antibody was from Trans Genic Inc (Hyogo, Japan).

Cell Culture. HEK293 cells were grown in Minimum Essential Medium supplemented with 10% fetal calf serum at 37°C under 5% CO_2 in air.

Detection of GSH. GSH concentration in HEK293 cells was determined using ThioGlo-1 (Calbiochem, San Diego, CA), a maleimide reagent that produces a highly fluorescent adduct upon reaction with thiol groups. GSH content was estimated from the fluorescence response via the interaction of ThioGlo-1 mainly with intracellular GSH. Cells were incubated at 37°C for 30 min with 10 μM ThioGlo-1. After washing with phosphate-buffered saline to remove excess non-reacted ThioGlo-1, the level of fluorescence was measured using a Multimode Detector DTX800 (Beckman Coulter, Fullerton, CA).

Transfection of GTRAP3-18 Antisense Oligonucleotides. HEK293 cells were transiently transfected with sense (GTGAACCTTGCCCCGCTC) or antisense (GAGCGGGGCAAGGTTCAC) GTRAP3-18 oligonucleotides using SuperFect™ (QIAGEN, Valencia, CA) as described previously (Watabe et al., 2004).

Immunoblot Analysis. Immunoblotting was performed as described previously (Watabe et al., 1996). Cells were lysed in buffer containing SDS and mercaptoethanol, and the cell lysate was then boiled. Denatured proteins were separated on polyacrylamide gel and transferred to a polyvinylidene difluoride membrane

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(Amersham-Pharmacia Biotech, Buckinghamshire, UK). The membrane was incubated with a blocking solution (2% bovine serum albumin dissolved in phosphate-buffered saline containing 0.2% Tween 20) for 1 h at room temperature and incubated with a first antibody dissolved in blocking solution overnight at 4°C. After washing, the membrane was incubated for 1 h with horseradish-linked secondary antibody. Immunoreactive proteins were detected with an enhanced chemiluminescence system (Amersham-Pharmacia Biotech, Buckinghamshire, UK). Band intensities were measured using Scion Image release Beta 4.0.3 (Scion Corporation, Frederick, MD).

Cellular cholesterol assay. The cells were extracted by sonication with 1% Triton X-100 in chloroform. After centrifugation at 10,000 × g for 10 min, organic phase was collected and dried. Dried lipids were used for measurement. Cholesterol assay was measured using a cholesterol quantitation kit (Bio Vision, Mountain View, CA) according to the manufacturer's directions except that cholesterol esterase was omitted from reaction mixture.

Quantification of DNA Fragmentation. DNA fragmentation was measured using a Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics, Indianapolis, IN) as described previously (Watabe and Nakaki, 2004).

Immunofluorescence Microscopy. As described previously (Watabe et al., 2000), cells were washed with phosphate-buffered saline and fixed with 3.7% formaldehyde for 20 min. Cells were permeabilized with phosphate-buffered saline containing 0.2% Triton X-100 for 5 min and then washed three times with phosphate-buffered saline. Incubation with primary antibody was carried out for 1 h at room temperature. Excess antibody was washed out three times with phosphate-buffered saline. This was followed by incubation with an appropriate fluorophore-labeled secondary antibody for 1 h at room temperature in an area shielded from light. After washing out the excess antibody three times with phosphate-buffered saline, coverslips were mounted using a ProLong Antifade Kit (Molecular Probes, Eugene, OR). Fluorescent images were

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obtained using a Zeiss fluorescence microscope (Zeiss, Oberkochen, Germany) and a BioRad inverted laser-scanning fluorescent microscope MRC-1024 using Laser Sharp 2000 software (BioRad, Tokyo, Japan).

Cell Surface Biotinylation. Labeling of proteins on the plasma membrane was accomplished by cell surface biotinylation using a Pinpoint^{TM} Cell Surface Protein Isolation Kit (Pierce, Rockford, IL) in accordance with the manufacturer's instructions.

Statistics. Values are mean ± S.E. from three experiments. Statistical analysis of the data was performed using analysis of variance followed by Fisher's test. A p value < 0.05 was considered significant.

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Results

We first ascertained whether GSH content is dependent on EAAC1 in HEK293 cells using EAAT inhibitors. The non-specific EAAT inhibitor DL-threo-β-benzyloxyaspartate (TBOA) dose-dependently decreased the intracellular GSH content (Fig. 1A). The GLT-1 specific inhibitor dihydrokainate (DHK) failed to decrease the intracellular GSH content at a concentration sufficient to inhibit GLT-1-mediated glutamate uptake (Fig. 1B). L-aspartate-β-hydroxamate (LAβH), which inhibits both GLT-1 and EAAC1, decreased the intracellular GSH content (Fig. 1C). Furthermore, the cystine transporter inhibitor quisqualate failed to decrease the intracellular GSH content (Fig. 1D). Therefore, EAAC1 mediates cysteine uptake for GSH synthesis in the cells.

We next examined the effect of intracellular GTRAP3-18 level on cellular GSH content. To increase GTRAP3-18 expression, the cells were treated with methyl-β-cyclodextrin (MeβCD), which increases GTRAP3-18 expression (Butchbach et al., 2003). As shown in Fig. 2A, GTRAP3-18 expression was examined using anti-GTRAP3-18 specific antibody. MeβCD increased the level of endogenous GTRAP3-18 protein in cells, as expected (Fig. 2B *left*). To decrease GTRAP3-18 expression, we used GTRAP3-18 antisense oligonucleotides and observed a specific reduction in the level of endogenous GTRAP3-18 protein (Fig. 2B *right*). The EAAC1 protein level was not affected by MeβCD or GTRAP3-18 antisense oligonucleotide treatment (Fig. 2C). Under these experimental conditions, intracellular GSH was increased concomitantly with a reduction in GTRAP3-18 protein level, while GSH content was decreased concomitantly with an increase in GTRAP3-18 protein level (Fig. 2D). When MeβCD-increased GTRAP3-18 was decreased by GTRAP3-18 antisense oligonucleotides, the GSH level was restored (Fig. 2E). MeβCD has a high affinity for cholesterol and has been shown to promote the efflux of cholesterol from cells (Kilsdonk et al., 1995). To examine whether the efflux of cholesterol caused the decrease in the

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GSH content, we quantitated the cholesterol content. The cholesterol content was slightly reduced by MeβCD at the concentration that increased GTRAP3-18 amount (Fig. 3A). However, GTRAP3-18 antisense oligonucleotides, which restored the GSH level decreased by MeβCD, did not affect the cholesterol content as compared with GTRAP3-18 sense oligonucleotides (Fig. 3B). This result indicates that the GSH level was not reduced by cholesterol efflux but by MeβCD-elevated GTRAP3-18. Moreover, an increased level of GTRAP3-18 rendered the cells more vulnerable to oxidative stress such as hydrogen peroxide (Fig. 4A), and a decreased level of GTRAP3-18 rendered the cells more resistant to hydrogen peroxide (Fig. 4B). These results show that GTRAP3-18 negatively regulates the intracellular GSH content, which in turn affects susceptibility to oxidative stresses such as hydrogen peroxide.

Immunocytochemical analysis revealed that GTRAP3-18 in control cells was present in both the plasma membrane and the intracellular compartment, and colocalized with EAAC1 (Fig. 5). In MeβCD-treated cells, GTRAP3-18 immunoreactivity was augmented in both the cell membrane and the intracellular compartment, while MeβCD did not alter the expression of EAAC1 protein (Fig. 5A). Cell surface EAAC1-associated GTRAP3-18 was increased by MeβCD. We ascertained that cell surface EAAC1-associated GTRAP3-18 was increased by MeβCD using another technique, a cell surface biotinylation assay. There was an increase in not only non-surface (non-biotinylated) but also surface (biotinylated) GTRAP3-18 level by MeβCD (Fig. 6). Protein kinase C activation is known to positively regulate cell surface expression of EAAC1 and activation of glutamate uptake by EAAC1 (Gonzalez et al., 2002; Gonzalez et al., 2003; Fournier et al., 2004). Therefore, we examined the effect of protein kinase C activation on GTRAP3-18 expression and GSH level in MeβCD-treated cells. PMA, a protein kinase C activator, induced an increase in cell surface EAAC1 level (Figs. 5 and 6). GSH content was elevated concomitantly with the increase in surface EAAC1 level by PMA, while 4α-PMA, which is inactive on

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protein kinase C, did not increase the GSH content (Fig. 7B). Since the inhibition of EAAC1 activity by LAβH suppressed the GSH content elevated by PMA, the PMA-elevated GSH content was mediated through EAAC1 activity (Fig. 7C and D). Treatment of MeβCD-treated cells with PMA induced a large increase in cell-surface-colocalized EAAC1 and GTRAP3-18 (Figs. 5A, B and 6). Importantly, however, the PMA-induced increase in GSH content was inhibited by the MeβCD-induced increase in cell surface GTRAP3-18 protein (Fig. 7).

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Discussion

Following the recent discovery of EAAC1 as a major neuroprotective molecule (Aoyama et al., 2006), we explored the mechanisms underlying the regulation of EAAC1 activity determining GSH content. Our results have demonstrated a cellular protein, GTRAP3-18, to dominantly and negatively regulate EAAC1 activity and determine the intracellular GSH content. In the brain, EAAC1 is the primary neuronal transporter among members of the EAAT family (Rothstein et al., 1994). To explore the mechanisms regulating EAAC1 activity on GSH content, we attempted to construct an *in vitro* neuronal EAAC1 model system. We judged that the use of a primary neuronal culture system would be unsuitable for our purpose because the expression of not only EAAC1 but also other EAATs, which are not expressed in neurons, is induced by preparation of a primary culture of neurons (Himi et al., 2003). Therefore, we used HEK293 cells, because they stably express only EAAC1 among members of the EAAT family (Lin et al., 2001), have markedly low ability to take up cystine via the cystine transporter (Shih and Murphy, 2001), are a cell line derived from humans, and are thus suitable as an *in vitro* neuronal EAAC1 model system. We used MeβCD to increase GTRAP3-18 expression. MeβCD increases endogenous GTRAP3-18 in HEK293 cells (Butchbach et al., 2003). Our study showed that MeβCD reduced the GSH content *via* an increase in GTRAP3-18. Moreover, this increased GTRAP3-18 rendered the cells more vulnerable to oxidative stress. MeβCD has high affinity for cholesterol and has been shown to promote efflux of cholesterol from the cell (Kilsdonk et al., 1995). There is no evidence for an association of EAAC1 with cholesterol efflux (Butchbach et al., 2004), and the mechanisms underlying the MeβCD-induced increase in GTRAP3-18 are poorly understood. We examined the change in cholesterol content under our experimental condition and found that the reduction of cholesterol content by MeβCD was slight. Previous reports suggest that the MeβCD concentration required to increase GTRAP3-18 is lower than that required to cause cholesterol depletion (Kilsdonk et al.,

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1995); (Butchbach et al., 2004). Moreover, we showed that GSH was reduced by MeβCD-increased GTRAP3-18 but not by cholesterol efflux. On the other hand, we used an antisense oligonucleotide technique to downregulate GTRAP3-18 expression rather than short interfering RNA, which can produce serious "off target" consequences. GTRAP3-18 antisense oligonucleotides specifically reduced endogenous GTRAP3-18 protein level and concomitantly increased intracellular GSH. This decreased level of GTRAP3-18 rendered the cells more resistant to hydrogen peroxide. These results clearly show GTRAP3-18 to negatively regulate the intracellular GSH content, which in turn affects susceptibility to oxidative stresses such as hydrogen peroxide.

Morphine has long been known to diminish intracellular GSH (Roberts et al., 1987), but the mechanisms underlying this GSH depletion are unknown. A murine homologue of GTRAP3-18 was identified as a factor that is upregulated in the basomedial amygdala in repeatedly morphine-administered mice (Ikemoto et al., 2002). Based on our results, the morphine-induced GSH depletion is possibly caused by induction of GTRAP3-18 on the plasma membrane, resulting in negative regulation of EAAC1.

Each EAAT family member is chemically modified by various stimuli. Oxygen radicals and hydrogen peroxide induce persistent inhibition of EAATs, probably *via* direct interaction with the transport process (Volterra et al., 1994). Nitric oxide generators such as sodium nitroprusside and S-nitroso-N-acetylpenicillamine decrease glutamate uptake into the synaptosome (Pogun et al., 1994). Peroxynitrite, formed by the combination of superoxide anion and nitric oxide, inhibits glutamate uptake by neuronal transporter EAAC1 (Trotti et al., 1996). However, the functional significance of these chemical modifications of EAAC1 remains unknown. Another modification of EAAC1 involves phosphorylation. In a tumor cell line, the cell surface expression and activity of EAAC1 appear to be regulated by several phosphorylation pathways. A protein kinase C-mediated pathway is known to positively regulate cell surface expression and activation of glutamate uptake by EAAC1 (Danbolt, 2001; Gonzalez et

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al., 2002; Gonzalez et al., 2003; Fournier et al., 2004; Huang et al., 2006). In particular, Huang et al. reported EAAC1 to be regulated by protein kinase Cα. Protein kinase Cα belongs to a classic subtype activated by diacylglycerol, which is produced by phospholipase C (Newton, 2001). Gq-coupled receptors, among many G protein-coupled receptors such as α1 adrenergic receptors, M1 muscarinic receptors and H1 histaminergic receptors, cause activation of phospholipase Cβ *via* the α subunit of Gq, which is activated by its ligand binding (Zhou et al., 1994). Since Hsu et al. reported that epinephrine increased the GSH level (Hsu et al., 2005), activation of EAAC1 *via* phosphorylation by protein kinase C is possibly caused by activation of these Gq-coupled receptors, resulting in positive regulation of EAAC1 and GSH synthesis.

EAAC1 is mainly localized in the intracellular compartment, with about 20% in the plasma membrane (Nieoullon et al., 2006). Phosphorylation by protein kinase C induces translocation of EAAC1 from the intracellular compartment to the plasma membrane and expression of its function as an amino acid transporter. On the other hand, GTRAP3-18 is also present mainly in the intracellular compartment and partially at the plasma membrane *via* binding to EAAC1 (Lin et al., 2001). Therefore, we examined the effect of protein kinase C activation on both GTRAP3-18 expression and GSH level in MeβCD-treated cells. Confirming the findings of Lin et al., GTRAP3-18 in control cells was present in both the plasma membrane and the intracellular compartment, and colocalized with EAAC1 (Lin et al., 2001). We further demonstrated PMA, a protein kinase C activator, to induce an increase in cell surface EAAC1 level and a concomitant increase in GSH content. This result suggests that protein kinase C up-regulates not only glutamate but also cysteine uptake by EAAC1. Moreover, treatment of MeβCD-treated cells with PMA induced a large increase in cell-surface-colocalized EAAC1 and GTRAP3-18, and decreased the GSH content. Importantly, the PMA-induced increase in GSH content was inhibited by the MeβCD-induced increase in plasma membrane GTRAP3-18 protein. GTRAP3-18

associated with EAAC1 in the plasma membrane dominantly and negatively regulated cysteine uptake for GSH synthesis, and determined intracellular GSH content even if protein kinase C, which activates EAAC1, was activated. Recently, it was reported that the phosphorylation of serine 465 in EAAC1 by protein kinase C is important for both the increase in EAAC1 activity and redistribution to the plasma membrane (Huang et al., 2006). Lin et al. reported that GTRAP3-18 was identified by a yeast two-hybrid screen system using the C-terminal intracellular domain (arginine 438 – phenylalanine 524) of EAAC1 (Kanai and Hediger, 1992; Lin et al., 2001; Yernool et al., 2004). Therefore, serine 465 of EAAC1, which is phosphorylated by protein kinase C, is located within the binding domain for GTRAP3-18. This, together with our results, indicates that GTRAP3-18 inhibits EAAC1 activity by masking the serine 465 residue, which is the site of phosphorylation by protein kinase C. Therefore, it is possible that a putative inhibitory compound against GTRAP3-18 would be an efficient GSH-increasing agent.

Since the GSH content in discrete brain areas is reduced in patients with Parkinson and Alzheimer disease (Dringen and Hirrlinger, 2003), GTRAP3-18 is a potential therapeutic target for increasing the neuronal GSH level. The discovery of a GTRAP3-18 inhibitory compound that increases neuronal GSH would contribute to developing novel therapeutic strategies to protect neurons in patients with neurodegenerative disorders including Parkinson and Alzheimer disease.

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Legends for figures

Figure 1. Effects of inhibitors of EAAT and cystine transporter on GSH content in HEK293 cells. After the cells had been treated with TBOA (A), DHK (B), LAβH (C) or quisqualate (D) at the indicated concentrations for 3 h, GSH (GSH) assay was performed. *, $p < 0.05$ compared with control.

Figure 2. Effects of MeβCD and GTRAP3-18 antisense oligonucleotides on GTRAP3-18 expression and GSH content in HEK293 cells. (A) After cell lysate preparation, immunoblot analysis was performed using anti-GTRAP3-18 specific antibody. (B) The cells were treated with MeβCD at the indicated concentrations, or transiently transfected with GTRAP3-18 antisense or sense oligonucleotides at the indicated concentrations. Two days later, immunoblot analysis was performed. (C) The cells were transiently transfected with 10 μM GTRAP3-18 sense (S) or antisense (As) oligonucleotides, or treated with 250 μM MeβCD. Two days later, immunoblot analysis was performed. (D) The cells were transiently transfected with 10 μM GTRAP3-18 sense (S) or antisense (As) oligonucleotides and then treated with 250 μM MeβCD. Two days later, GSH assay was performed. *, $p < 0.05$ compared with control. (E) After the cells had been transiently transfected with 10 μM GTRAP3-18 sense (Sense) or antisense (Antisense) oligonucleotides, cells were treated with 250 μM MeβCD. Two days later, GSH assay was performed.

Figure 3. Effects of MeβCD and GTRAP3-18 antisense oligonucleotides on cholesterol content in HEK293 cells. (A) After the cells had been treated with MeβCD at the indicated concentrations for 2 days, cholesterol assay was performed. (B) After the cells had been transiently transfected with 10 μM GTRAP3-18 sense (Sense) or antisense (Antisense) oligonucleotides, cells were treated with 250 μM MeβCD. Two days later, cholesterol assay was performed.

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Figure 4. Effects of MeβCD and GTRAP3-18 antisense oligonucleotides on hydrogen peroxide-induced apoptosis in HEK293 cells. (A) After the cells had been treated with 250 μM MeβCD for 2 days, they were treated with hydrogen peroxide at the indicated concentrations for 24 h and DNA fragmentation assay was performed. (B) The cells were transiently transfected with 10 μM GTRAP3-18 sense (S) or antisense (As) oligonucleotides. Two days later, they were treated with 75 μM hydrogen peroxide for 24 h and DNA fragmentation assay was performed. *, $p < 0.05$ compared with control.

Figure 5. Immunofluorescence microscopy showing changes in EAAC1 and GTRAP3-18 localization by MeβCD and PMA treatment of HEK293 cells. The cells were treated with 250 μM MeβCD for 2 days and then with 1 μM PMA for 3 h. Intracellular localization of EAAC1 (green) and GTRAP3-18 (red) was examined by immunofluorescence microscopy using a fluorescent microscope (A) and a confocal laser-scanning fluorescent microscope (B).

Figure 6. Biotinylation showing changes in EAAC1 and GTRAP3-18 on cell surface by MeβCD and PMA treatment of HEK293 cells. Cells were treated with 250 μM MeβCD for 2 days and then with 1 μM PMA for 3 h. Following biotinylation of intact cells, immunoblotting of biotinylated (cell surface) and non-biotinylated (intracellular) fractions were performed using each specific antibody. Actin was measured to determine the degree of intracellular protein labeling by the biotin reagent.

Figure 7. Changes in GSH content by MeβCD and PMA treatment of HEK293 cells. (A) After the cells had been treated with MeβCD at the indicated concentrations for 2 days and with 1 μM PMA for 3 h, GSH assay was performed. (B) After the cells

had been treated with 250 μM MeβCD for 2 days and with PMA or 4α-PMA at the indicated concentrations for 3 h, GSH assay was performed. (C) After pretreatment with LAβH at the indicated concentrations, the cells were treated with 1 μM PMA for 3 h and GSH assay was performed. (D) After pretreatment with 250 μM LAβH, the cells were treated with PMA at the indicated concentrations for 3 h and GSH assay was performed.

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Figure 1

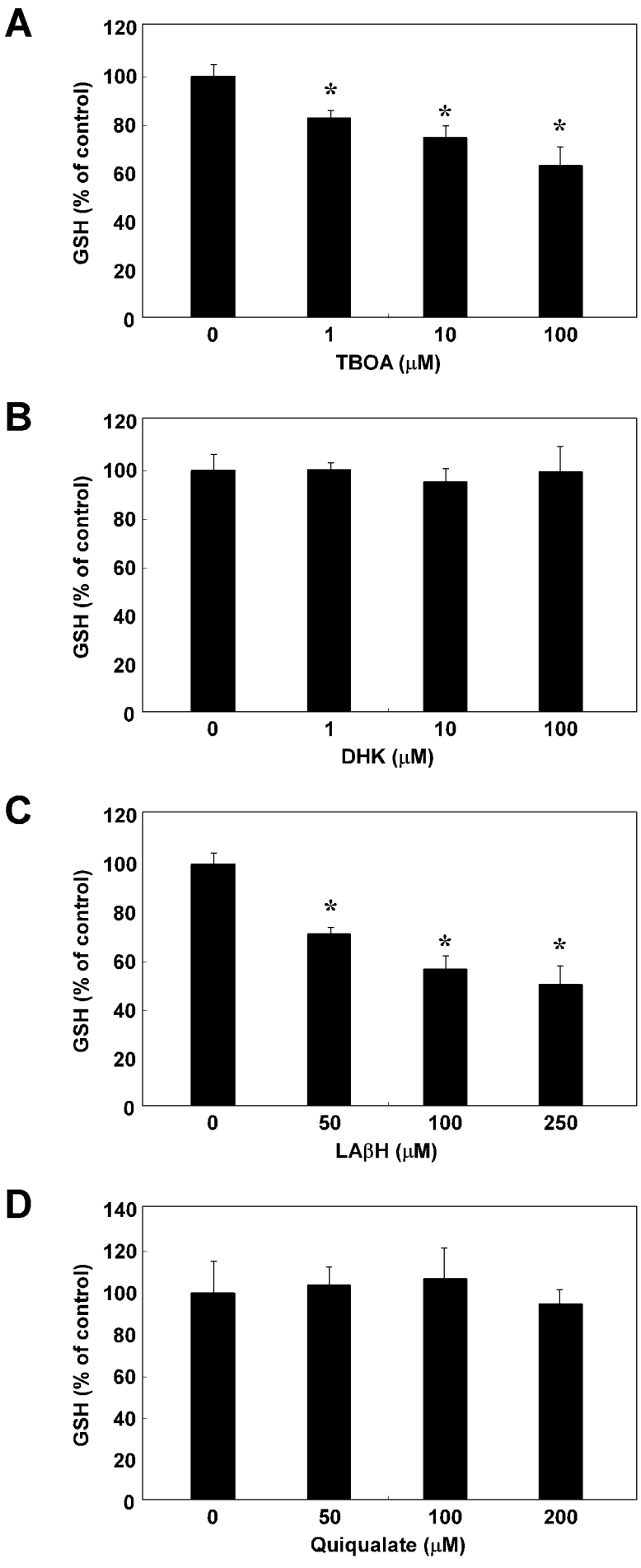

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Figure 2

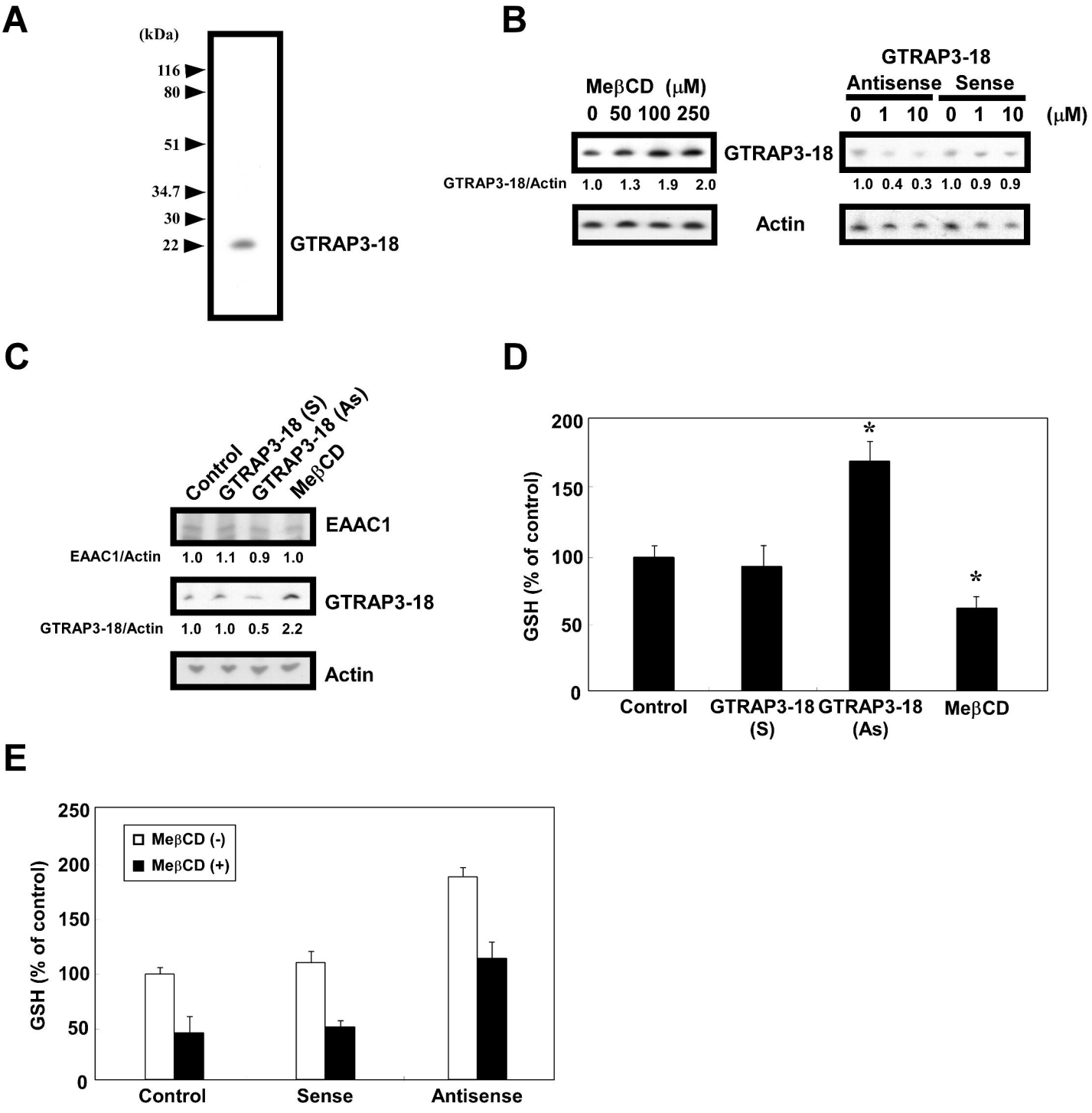

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Figure 3

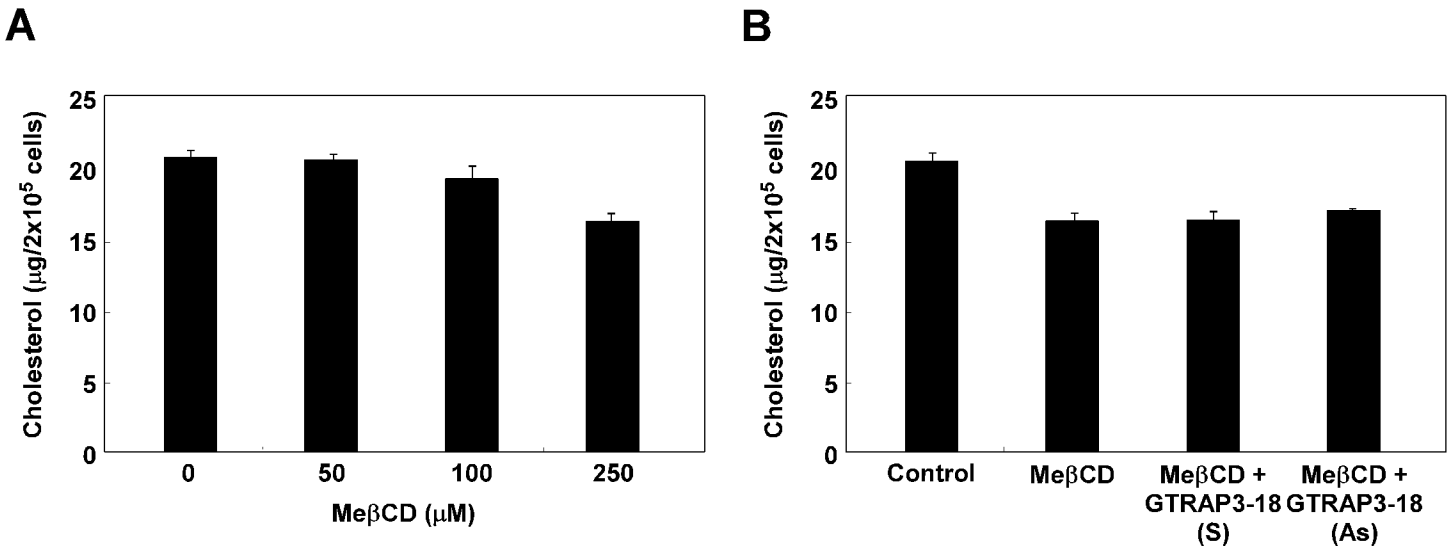

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Figure 4

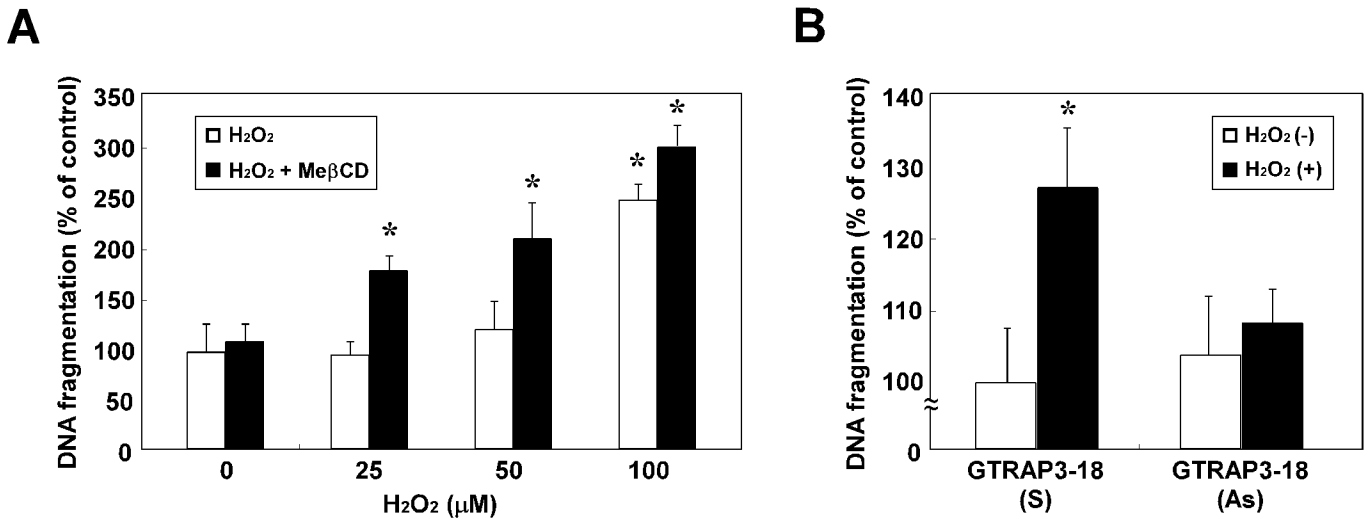

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Figure 5

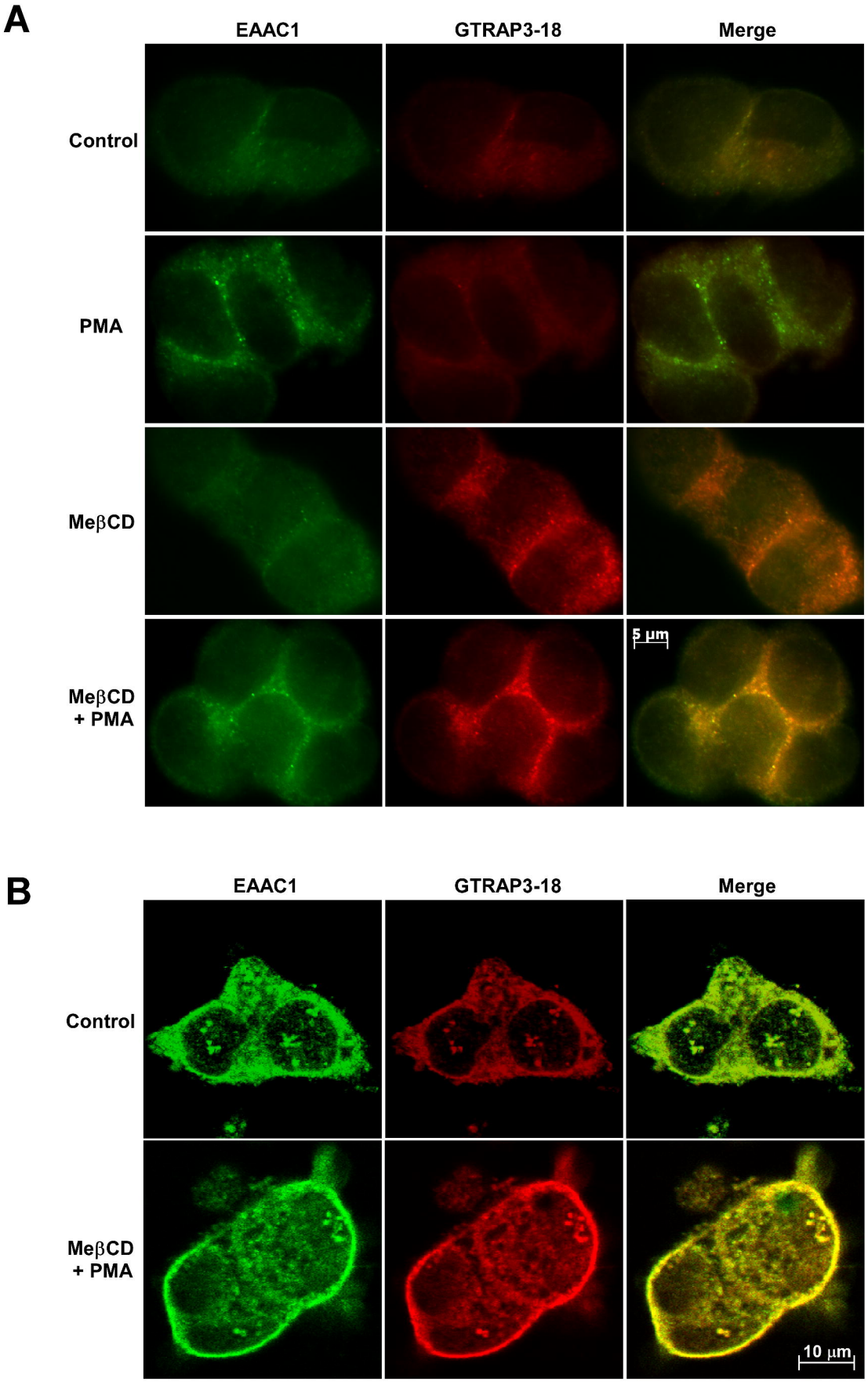

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Figure 6

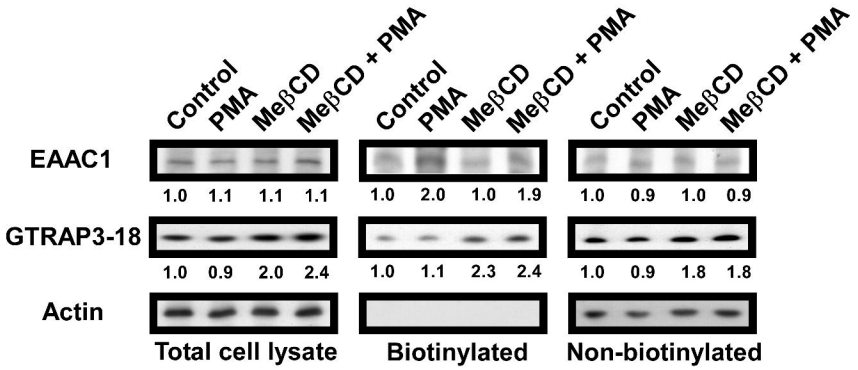

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Figure 7