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Estrogen Receptor β -Mediated Nuclear Interaction Between IRS-1 and Rad51 Inhibits Homologous Recombination Directed DNA Repair in Medulloblastoma

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Abstract

In medulloblastomas, which are highly malignant cerebellar tumors of the childhood genotoxic treatments such as cisplatin or γ -irradiation are frequently associated with DNA damage, which often associates with unfaithful DNA repair, selection of new adaptations and possibly tumor recurrences. Therefore, better understanding of molecular mechanisms which control DNA repair fidelity upon DNA damage is a critical task. Here we demonstrate for the first time that estrogen receptor beta (ER β) can contribute to the development of genomic instability in medulloblastomas. Specifically, ER β was found highly expressed and active in mouse and human medulloblastoma cell lines. Nuclear ER β was also present in human medulloblastoma clinical samples. Expression of ER β coincided with nuclear translocation of insulin receptor substrate 1 (IRS-1), which was previously reported to interfere with the faithful component of DNA repair when translocated to the nucleus. We demonstrated that ER β and IRS-1 bind each other, and the interaction involves C-terminal domain of IRS-1 (aa 931–1233). Following cisplatin-induced DNA damage, nuclear IRS-1 localized at the sites of damaged DNA, and interacted with Rad51—an enzymatic component of homologous recombination directed DNA repair (HRR). In medulloblastoma cells, engineered to express HRR-DNA reporter plasmid, ER antagonist, ICI 182,780, or IRS mutant (931–1233) significantly increased DNA repair fidelity. These data strongly suggest that both molecular and pharmacological interventions are capable of preventing ER β -mediated IRS-1 nuclear translocation, which in turn improves DNA repair fidelity and possibly counteracts accumulation of malignant mutations in actively growing medulloblastomas.

Insulin receptor substrate 1 (IRS-1) is a 160 kDa cytosolic protein implicated in insulin receptor and IGF-I receptor (IGF-IR) signaling (Baskin et al., 1994; Myers et al., 1994). Besides its metabolic and growth promoting function, IRS-1 is suspected to play a role in malignant transformation, presumably by amplifying the IGF-IR signal. The first convincing evidence indicating the transforming potential of IRS-1 was demonstrated in R⁺ cells, which are mouse embryo fibroblasts from mice with targeted disruption of the IGF-IR gene. R⁺ cells, which are resistant to SV40 T-antigen-mediated transformation, acquired a transformed phenotype

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following co-transfection with IRS-1 and T-antigen containing expression vectors (Sell et al., 1993; D'Ambrosio et al., 1995). In a similar manner, T-antigen from human polyomavirus JC (JCV T-antigen) also required the presence of the IGF-IR-IRS-1 signaling axis for transformation (Del Valle et al., 2002b). Interestingly, we have found nuclear IRS-1 in cells expressing JCV T-antigen, and in JCV T-antigen positive medulloblastoma clinical samples (Del Valle et al., 2002b; Lassak et al., 2002; Trojanek et al., 2006a). This first demonstration of nuclear IRS-1 was further confirmed by others. For example, nuclear IRS-1 has been found in cells transfected with v-src and SV40 T-antigen (Prisco et al., 2002; Tu et al., 2002, 2003), in fibroblasts stimulated with IGF-I (Prisco et al., 2002), in hepatocytes (Boylan and Gruppiso, 2002), in 32D cells (Tu et al., 2003), and in osteoblasts (Seol and Kim, 2003). More recently, nuclear IRS-1 was detected in breast cancer cells in association with estrogen receptor alpha (ER α) (Morelli et al., 2004). Although the mechanism by which IRS-1 is translocated to the nucleus is not well established, it could utilize its own putative nuclear localization signal (Tu et al., 2002). On the other hand, the abundant presence of nuclear IRS-1 in cells expressing proteins with high affinity to the nucleus, such as JCV T-antigen, SV40 T-antigen and ER α , may indicate that IRS-1 requires a chaperone for efficient nuclear translocation. The biological relevance of nuclear IRS-1 has been studied extensively during last several years. In fibroblasts stimulated with IGF-I, nuclear IRS-1 has been found in association with upstream binding factor (UBF1—regulator of RNA polymerase I), which coincided with increased rRNA synthesis (Drakas et al., 2004). In addition, a direct connection between IRS-1 and homologous recombination dependent DNA repair (HRR) has recently been postulated (Trojanek et al., 2003). This new signaling interaction involves perinuclear binding between hypo-phosphorylated IRS-1 and the major enzymatic component of HRR, Rad51. IGF-I-mediated IRS-1 tyrosine phosphorylation attenuated IRS-1 binding to Rad51 allowing efficient translocation of Rad51 to the sites of damaged DNA, which lead to the increased contribution of HRR in DNA repair of double strand breaks. In JCV T-antigen positive cells however, IRS-1 translocates to the nucleus, where it has been found in a complex with Rad51 (Trojanek et al., 2006a,c). Since the nuclear presence of IRS-1 was also demonstrated in the absence of T-antigen, in ER α positive breast cancer cells (Morelli et al., 2004), we asked whether ER-mediated IRS-1 nuclear translocation could inhibit HRR in medulloblastoma tumors, which are JCV negative. This could be quite relevant for medulloblastoma, since we have detected constitutively active ER β in all medulloblastoma cell lines examined, and in over 30% of medulloblastoma clinical samples. The results presented in this report indicate the involvement of ER β in the inhibition of the faithful component of DNA repair, HRR. The suspected mechanism involves the formation of an ER β –IRS-1 complex in the nucleus, nuclear interaction between IRS-1 and Rad51, and the inhibition of HRR. This could represent the major driving mechanism for genomic instability and the development of mutagenic phenotype after genotoxic treatment, which may act independently or in synergy with JC polyomavirus large T-antigen.

Materials and Methods

Immunohistochemistry

A total of 19 cases of human medulloblastoma were acquired from the pathology archives of the University Health Network, University of Toronto, Ontario, Canada. The formalin-fixed, paraffin embedded samples were sectioned at 4 μ m in thickness, stained with Hematoxylin and Eosin and histologically classified according to the World Health Organization Classification of Tumors of the Nervous System. Immunohistochemistry was performed using the avidin–biotin–peroxidase complex system, according to the manufacturer's instructions (Vectastain Elite ABC Peroxidase Kit; Vector Laboratories, Burlingame, CA). Briefly, sections were deparaffinized in xylene and re-hydrated through descending alcohols to water. For non-enzymatic antigen retrieval sections were heated in 0.01 M sodium citrate buffer (pH 6.0) to

95°C for 40 min and allowed to cool for 20 min at room temperature. To quench endogenous peroxidase, slides were then rinsed with PBS and incubated in MeOH/3% H₂O₂ for 20 min. Sections were then washed with PBS and blocked in PBS/0.1% BSA containing 5% normal horse or goat serum for 2 h at room temperature, and incubated overnight at room temperature with primary antibodies. The primary antibodies used in this study include: anti-IRS-1 rabbit polyclonal (1:500) (UBI, Lake Placid, NY), and anti-ER β rabbit polyclonal (1:500) (H-150; Santa Cruz, Santa Cruz, CA) antibodies. Secondary antibody used was biotin-conjugated goat anti-rabbit IgGs (Vector Laboratories). Sections were developed with a diaminobenzidine substrate (Sigma, St. Louis, MO), counterstained with hematoxylin and mounted with Permount. For double immunohistofluorescent labeling, ER β was detected by mouse anti-ER β monoclonal antibody (1:100) (C14C8; GeneTex, San Antonio, TX) followed by FITC-conjugated goat anti-mouse secondary antibody (Molecular Probes, Inc., Eugene, OR). IRS-1 was detected with rabbit anti-IRS-1 antibody (UBI), which recognizes the C-terminal portion of the IRS-1 protein, and rhodamine-conjugated goat anti-rabbit secondary antibody (Molecular Probes, Inc.).

Cell culture

The following cell lines were used in this study: mouse medulloblastoma, JCV T-antigen positive BsB8 and JCV T-antigen negative Bs-1a, which were both isolated from a cerebellar tumor of a transgenic mouse expressing the early genome of human polyomavirus JC (Krynska et al., 1999; Wang et al., 2001); and two human medulloblastoma cell lines, D384 Med, and Daoy. D384 are metastatic medulloblastomas isolated from peritoneal ascites of a child with medulloblastoma (ATCC#: HTB-185), and Daoy derive from a tumor in the posterior fossa of a 4-year-old boy (ATCC# HTB186). BsB8 cells are very aggressive, capable of forming large subcutaneous and intracranial tumors following stereotactic injection of a small number of cells in to the brain, and have the ability of spreading into cerebellum (Reiss, 2002; Wang et al., 2004). BsB8 and Daoy were maintained as monolayer cultures in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO-BRL, Grand Island, NY) containing 10% fetal bovine serum (FBS), at 37°C in a 7% CO₂ atmosphere. D384 were cultured in suspension, in the Eagle's MEM supplemented with non-essential amino acids (GIBCO-BRL), 2 mM L-glutamine, 1 mM sodium pyruvate, and 20% FBS. The cells were made partially quiescent by 48 h incubation in serum-free medium (SFM) (DMEM supplemented with 0.1% bovine serum albumin). Cellular growth and signaling responses were tested by stimulating serum starved cells with 50 ng/ml of recombinant IGF-I (GIBCO-BRL).

Luciferase assay

The ER transcriptional activity in BsB8 Daoy and D384 cells was determined by utilizing the ERE-LUC reporter plasmid, which contains the luciferase gene driven by ER responsive element (ERE) (Morelli et al., 2004). The activation of ERE was evaluated by a dual-Firefly/Renilla luciferase reporter system (Promega, Madison, WI) using Fentomaster FB12 chemiluminometer (Zylux Corp., Huntsville, AL).

Western blot and immunoprecipitation

To isolate protein extracts, monolayer cultures were lysed for 5 min on ice with 400 μ l of lysis buffer A [50 mM HEPES; pH 7.5; 150 mM NaCl; 1.5 mM MgCl₂; 1 mM EGTA; 10% glycerol; 1% Triton X-100; 1 mM phenylmethylsulfonyl fluoride (PMSF); 0.2 mM Na-orthovanadate and 10 μ g/ml aprotinin]. In some cases, to improve recovery of DNA bound proteins (Rad51), DNase was added to the lysis buffer. Total proteins (50 μ g) were separated on a 4–15% gradient SDS-PAGE (BioRad, Hercules, CA). The resulting blots were probed with following primary antibodies: anti-Rad51 rabbit polyclonal, (Ab-1, Oncogene, Boston, MA); anti-ER β rabbit polyclonal (Santa Cruz), anti-ER α rabbit polyclonal (Santa Cruz), anti-IRS-1 rabbit polyclonal

(UBI). Anti-Grb-2 antibody (Transduction Laboratories, Lexington, KY), was used to monitor equal loading conditions (Trojanek et al., 2003). For immunoprecipitation/Western blot experiments (IP/W) cell fractionation procedure was applied to separate cytoplasmic and nuclear proteins using the Nuclear/Cytosol Fractionation kit (BioViosion, Mountain View, CA) with some modifications (Trojanek et al., 2003). IRS-1, Rad51 and ER β were immunoprecipitated from 500 μ g of nuclear extracts with, anti-Rad51 mouse monoclonal (clone 3C10; UBI), anti-ER β mouse monoclonal (GeneTex), or anti-IRS-1 mouse monoclonal (UBI) antibodies, utilizing agarose-conjugated anti-mouse and anti-rabbit antibodies (Sigma). To avoid the use of the same type of antibody for IP and Western blot, corresponding blots were developed with anti-IRS-1 rabbit polyclonal antibody (UBI), anti-Rad51 rabbit polyclonal antibody, and anti-ER β rabbit polyclonal antibody (Santa Cruz).

Immunocytofluorescence

All cells were cultured on poly-D-lysine coated Lab-Tek Chamber Slides (Nalge Nunc International, Rochester, NY). Cisplatin treatment, 1 μ g/ml, was applied to exponentially growing cells for a period of 6 h. For immunostaining cells were fixed and permeabilized with the buffer containing 0.02% Triton X-100 and 4% formaldehyde in PBS. Fixed cells were washed 3 $\times$ in PBS and blocked in 1% BSA for 1 h at 37°C. RAD51 was detected by mouse anti-RAD51 monoclonal antibody (UBI) followed by FITC-conjugated goat anti-mouse secondary antibody (Molecular Probes, Inc.). IRS-1 was detected with rabbit anti-IRS-1 antibody (UBI), which recognizes the C-terminal portion of the IRS-1 protein, and rhodamine-conjugated goat anti-rabbit secondary antibody (Molecular Probes, Inc.). ER β was detected by anti-ER β mouse monoclonal antibody (GeneTex) and FITC-conjugated goat anti-mouse secondary antibody (Molecular Probes, Inc.). Finally, phospho-histone H2AX was detected by a mouse monoclonal antibody which recognizes phosphorylated serine within the 134–142 aa fragment of human histone H2AX (UBI), and rhodamine-conjugated goat anti-mouse secondary antibody (Molecular Probes, Inc.). Negative controls were routinely performed in the presence of an irrelevant, anti-BrdU antibody instead of primary antibodies. The images were visualized with an inverted Olympus 1 $\times$ 70 fluorescence microscope equipped with a Cook Sensicom ER camera (Olympus America, Inc., Melville, NY). A series of three-dimensional images of each individual picture were deconvoluted to one two-dimensional picture and resolved by adjusting the signal cut-off to near maximal intensity to increase resolution. Final pictures were prepared with Adobe Photoshop to demonstrate subcellular localization and co-localization between detected proteins.

Homologous recombination directed DNA repair (HRR)

The plasmid pDR-GFP (generously provided by Dr. M. Jasin, Sloan-Kettering Cancer Center, New York) was stably introduced, by calcium phosphate reagent (Promega), into Daoy cells. Stable clones were selected in puromycin (2 μ g/ml) and characterized by Southern blot. The pDRGFP contains a non-active gene for GFP (SceGFP) as a recombination reporter, and a fragment of the GFP gene as a donor for homologous repair (Pierce et al., 1999). The SceGFP cassette has an inactivating insertion, which consists of two STOP codons and a restriction site for the rare cutting endonuclease, I-SceI. When I-SceI is expressed in DR-GFP expressing clones, it inflicts DSBs within the SceGFP fragment providing a signal for homologous recombination and the reconstruction of functional GFP. To analyze the effectiveness of HRR, cells were transiently transfected with 3 μ g of pC β A-Sce and 1 μ g of pDsRed1-Mito (Clontech, Palo Alto, CA) utilizing Fugene6 reagent (Roche, Indianapolis, IN). pC β A-Sce contains I-SceI cDNA to generate DSBs in SceGFP cDNA (Pierce et al., 1999) and pDsRed1-Mito contains red fluorescent protein with a mitochondrial localization signal to control for the efficiency of transfection. DNA repair by HRR was evaluated by counting cells with both green nuclear fluorescence and red mitochondrial fluorescence versus all positively transfected cells (only red cells + red and green cells) at 96 h after transfection. Cells showing green nuclear

fluorescence only were sporadically detected; however these cells were not considered in the calculation.

Results

Occurrence of ER β expression in medulloblastoma clinical samples

The results in Table 1 indicate that 15 out of 19 medulloblastoma clinical samples were positive for IRS-1, and among these 15 positive cases, 5 were characterized by a strong nuclear IRS-1 immunolabeling, and in an additional 4 cases, IRS-1 was detected both in the nuclei and in the cytoplasm. We have also detected nuclear ER β in 6 out of 19 medulloblastoma cases, 3 of which were neuroblastic, 2 desmoplastic, and 1 classic medulloblastoma, and indeed, these 6 cases were characterized by a strong nuclear presence of IRS-1. The results presented in Figure 1 show IRS-1 and ER β immunohistolabeling in normal human cerebellum, and in one ER β -negative and one ER β -positive example of medulloblastoma. In the absence of ER β , we found a strong cytosolic immunolabeling with anti-IRS-1 antibody. However, in the ER β -positive tumors, the labeling pattern of IRS-1 changed from cytosolic to nuclear in a large number of cells (brown nuclei). Note however that a substantial number of cells within the tumor were negative for nuclear IRS-1 (blue nuclei). In control cerebellar tissue, a weak cytosolic labeling of IRS-1 was detected in granule neurons and in Purkinje cells (much weaker than in the tumor tissue). Surprisingly, the labeling of normal human cerebellum with anti-ER β antibody demonstrated the presence of ER β in the cytosol of Purkinje cells and in some granule neurons, which may indicate lack of ER β transcriptional activity in this control normal tissue. The results in Figure 2 show nuclear co-expression between IRS-1 and ER β (yellow fluorescence) in the clinical sample previously characterized by the nuclear presence of IRS-1 and ER β (sample #9 from Table 1). Although ER β –IRS-1 nuclear co-expression was detected in the majority of cells in the tumor tissue, there are single cells which demonstrated nuclear IRS-1 in the absence of nuclear ER β (red fluorescence) and cells with nuclear ER β in the absence of nuclear IRS-1 (green fluorescence).

As shown in Figure 3A, three human medulloblastoma cell lines (Daoy, D384, and D425), and two mouse medulloblastoma cell lines (Bs-1a and BsB8) are characterized by substantially higher levels of ER β than the levels detected in the human breast cancer cell line MCF7 (positive control for ER α and ER β). Interestingly, we did not detect ER α in human medulloblastoma cells, and found a weak band corresponding to ER α in one of the two mouse medulloblastoma cell lines (Bs-1a). As expected, medulloblastoma cell lines examined expressed also high levels of IRS-1 (Wang et al., 2001; Del Valle et al., 2002a). In addition, immunocytofluorescent labeling depicted in Figure 3B shows substantial amounts of ER β in the nuclei of exponentially growing mouse and human medulloblastoma cell lines, which could indicate a constitutive transcriptional activity of ER β these cells.

Therefore, we have utilized the reporter system in which estrogen responsive elements (EREs) were cloned in frame with luciferase cDNA (Morelli et al., 2004). The results in Figure 4 indicate that ERE background activity was high in exponentially growing human medulloblastoma cell lines (D384 and Daoy), and was only slightly elevated by 17 β -estradiol (E2). In contrast, ER α / β antagonist, ICI 182,780, significantly attenuated ER β -mediated transcriptional activity in the medulloblastoma cell lines examined. Although similar responses were observed in BsB8 cells, absolute values of the ERE activation were at least several-fold lower in comparison to human cell lines. This correlated well with much higher ER β protein levels detected in human medulloblastoma cell lines (compare Figs. 3 and 4).

ER β –IRS-1 interaction in human medulloblastoma cell lines

Since nuclear IRS-1 is suspected to interfere with DNA repair of double strand breaks (Reiss et al., 2006; Trojanek et al., 2006a,c), we have further evaluated the possibility of nuclear translocation of IRS-1 in T-antigen negative human medulloblastoma cell lines (D384 and Daoy), which express high levels of ER β . The results of reciprocal IP/Western shown in Figure 5A demonstrate that anti-ER β and anti-IRS-1 antibodies co-precipitated IRS-1 and ER β , respectively, from D384 and Daoy protein lysates, suggesting that IRS-1 and ER β form functional protein complexes. We have confirmed this protein-protein interaction by double immunocytofluorescence with anti-IRS-1 rabbit polyclonal antibody and anti-ER β mouse monoclonal antibody. The results in Figure 5B demonstrate the presence of IRS-1 (red) and ER β (green) in the nucleus (blue) of D384 and Daoy cells. The images in part B represent a single horizontal section (0.2 μ m) taken across the nucleus from the fluorescent microscope equipped with motorized Z-axis and deconvolution software (SlideBook4). Note the presence of a possible nuclear interaction (co-localization) between IRS-1 and ER β (yellow fluorescent foci). Interestingly, these co-localization nuclear foci were much more apparent when exponentially growing cells were pretreated with cisplatin (1 μ g/ml) for 6 h. This could indicate that IRS-1–ER β complexes may be formed in the vicinity of damaged DNA. Indeed, in cisplatin treated Daoy cells, double immunolabeling with anti-IRS-1 and anti-histone γ H2AX antibodies demonstrated the presence of IRS-1– γ H2AX co-localization complexes (Fig. 5C).

Since the results in Figure 5 indicate the presence of nuclear IRS-1 in association with ER β , it is possible that this nuclear IRS-1 could interfere with the process of DNA repair in which the DNA binding protein, Rad51, is involved. To test this possibility, we have exposed proliferating cultures of Daoy cells to cisplatin for 6 h, which induces DNA double strand breaks (DSBs) in cells replicating DNA (Wang et al., 2005). Double immunolabeling of these cisplatin treated cells with anti-Rad51 and anti-IRS-1 antibodies revealed a large number of nuclear foci in which IRS-1 co-localized with Rad51 (yellow fluorescence) (Fig. 6A). Quantitatively, more than 50% of Rad51 nuclear foci were occupied by nuclear IRS-1 (yellow foci) in exponentially growing Daoy cells treated with cisplatin (Fig. 6B). Following ER β inhibition by ICI 182,780, the number of Rad51 foci occupied by IRS-1 decreased from 50% to 26%, which was associated with a significant decrease in nuclear IRS-1 (red foci) (Fig. 6B). In control cells in which cisplatin treatment was omitted, Rad51 nuclear foci were rare, and only perinuclear co-localization complexes between IRS-1 and Rad51 were detected (not shown).

In addition, we have performed subcellular fractionation and isolated proteins from cytosolic and nuclear fractions of exponentially growing Daoy cells in the presence (FBS +ICI) and absence (FBS) of ER α / β antagonist, ICI 182,780. The results shown in Figure 6C show both nuclear and cytosolic presence of IRS-1 in Daoy cells growing in 10% FBS. The amount of IRS-1 present in the nucleus was strongly decreased in cells treated with ICI 182,780. The same Western blot membrane was re-probed with anti-ER β antibody, showing its predominant nuclear localization, and then with cytosolic marker, Grb-2, which verify purity of the nuclear fraction. We have confirmed nuclear co-localization between Rad51 and IRS-1 by precipitating Rad51 from nuclear extracts of Daoy cells cultured in FBS; FBS +ICI and FBS +E2 (Fig. 6D). Note a significant decrease in the intensity of the ER β –IRS-1 immunocomplex in cells treated with ICI 182,780.

Effects of ER β –IRS-1 complex on homologous recombination directed DNA repair (HRR)

Next we asked whether ER β interaction with IRS-1, and associated IRS-1 nuclear translocation could affect HRR. We have applied two strategies to interfere with the ER β –IRS-1 complexes. The first strategy is based on the treatment of cells with ER α / β antagonist, ICI 182,780, and the second utilizes a new IRS-1 mutant which should compete with the endogenous IRS-1 for

the binding of ER β . The results of GST-pull down assay shown in Figure 7A demonstrate that the domain of IRS-1 responsible for ER β binding is localized in the C-terminal portion of IRS-1, amino acids 931–1233. Since Rad51 binds N-terminal portion of IRS-1 (Trojanek et al., 2006a), we have generated the expression vector in which 931–1233 fragment of IRS-1 was cloned under the control of CMV promoter (Fig. 7B). This IRS-1 fragment is expected to translocate with ER β to the nucleus, however it should not interact with Rad51. Figure 7C demonstrates HRR activity assayed by the DRGFP reporter system developed by Maria Jasin (Pierce et al., 1999). In this assay, Daoy cells stably expressing the DRGFP reporter system have been utilized. Note that inhibition of ER β by ICI 182,780, as well as transient expression of the 931–1233 IRS-1 mutant (Fig. 7C), significantly increased the number of Daoy cells capable of repairing I-Sce-I inflicted DSBs by homologous recombination (Fig. 7D). This increased activity of HRR was accompanied by a significant decrease in Rad51 nuclear co-localization with IRS-1 in medulloblastoma cells in which the cisplatin treatment was applied in the presence of ICI 182,780 (compare Figs. 6B and 7D).

In summary, our results demonstrate for the first time elevated expression and constitutive activity of ER β in mouse and human medulloblastoma cell lines, and in human medulloblastoma clinical samples. Importantly, this enhanced ER β activity correlated with the detection of nuclear IRS-1, which interacted with Rad51 at the sites of damaged DNA and inhibited HRR.

Discussion

Our results demonstrate the presence of ER β in the nucleus in over 30% of medulloblastoma clinical samples, which was strongly associated with the detection of nuclear IRS-1 (Table 1). In addition, human and mouse medulloblastoma cell lines express high quantities of transcriptionally active ER β . We have demonstrated that both basic and estrogen-stimulated levels of HRR were relatively low in Daoy cells, which stably express the HRR reporter system (Daoy/DRGFP). In contrast, inhibition of ER β by ICI 182,780 activated this faithful component of DNA repair (Fig. 6C). We have also located the binding site for ER β within the C-terminal domain IRS-1 (Fig. 6A), and showed that this C-terminal fragment of IRS-1 can enhance HRR when expressed in exponentially growing cultures of medulloblastoma cells (Fig. 6C,D). This in turn could improve DNA repair fidelity, possibly counteracting the accumulation of spontaneous mutations and tumor recurrences after the treatment of medulloblastoma with genotoxic agents.

While strong experimental and clinical evidence supports the involvement of IGF-IR in medulloblastoma (Patti et al., 2000; Wang et al., 2001; Reiss, 2002; Del Valle et al., 2002a,b), the potential impact of the IGF-IR signaling system on the fidelity of DNA repair of double strand breaks (DSBs) represents a quite new concept (Trojanek et al., 2003, 2006a,c; Reiss et al., 2006). Until now, there have been only scattered reports suggesting that the IGF-IR may have functions affecting DNA repair. These include enhanced radioresistance, which was found proportional to the IGF-IR protein level in mouse embryo fibroblasts and breast tumor cells (Turner et al., 1997); enhanced DNA repair via the IGF-I activated p38 signaling pathway in response to UV-mediated DNA damage (Heron-Milhavet and LeRoith, 2002); and delayed UVB-induced apoptosis via IGF-I-mediated activation of Akt, resulting in enhanced repair of DNA cyclobutane thymidine dimers (Decraene et al., 2002). In contrast, one report suggested a delay in DNA repair of potentially lethal radiation damage observed in the presence of IGF-I and insulin treatments of A549 cells (Jayanth et al., 1995). Although these few reports indicate that the IGF-IR signaling may contribute to the development of drug resistance and/or DNA repair, it remains unclear whether the receptor affects DNA repair directly, or whether its strong anti-apoptotic properties (Sell et al., 1995; D'Ambrosio et al., 1997; Valentinis et al., 1998) simply increase the resistance to genotoxic agents. Recent results from our laboratory

demonstrate that activated IGF-IR can enhance HRR in fibroblasts (Trojanek et al., 2003) and in medulloblastoma cell lines (Trojanek et al., 2006a,b). The mechanism involves the major IGF-IR signaling molecule, IRS-1, which seems to control Rad51 translocation to the sites of damaged DNA. This effect is based on cytosolic interaction between Rad51 and IRS-1. The binding is direct, occurs within the perinuclear region of the cell, involves the N-terminal portion of IRS-1, and is negatively regulated by IGF-I-mediated IRS-1 tyrosine phosphorylation (Trojanek et al., 2003). Importantly, cells characterized by low levels of the IGF-IR, or cells expressing an IGF-IR mutant that fails to phosphorylate IRS-1, retain significant amounts of Rad51 within the perinuclear compartment, and show significantly less DNA repair by HRR (Trojanek et al., 2003).

Gonadal steroids are important regulators of neuronal development (Jakab et al., 2001). They function by activating nuclear proteins known as estrogen receptors (ERs). In the developing cerebellum, ER α and ER β mRNAs and proteins were detected in neurons and in some glial cells (Jakab et al., 2001). Importantly, detectable levels of ER β protein were found in granule and Purkinje neurons throughout normal cerebellar development (Belcher, 1999). In primary cultures of cerebellar granule neurons, 17 β -estradiol (E2) and ICI 182,780 were shown to affect the phosphorylation status of MAP kinases, differentially affecting cell proliferation and cell survival in this model (Wong et al., 2003). Although the role of ER α in several tumors, including breast, ovarian, prostate and colon cancer has been intensively studied, a potential function of ER β in malignant transformation is still unclear. In this respect, ER β protein has been detected in breast, ovarian, prostate and colon cancer, and in astrocytic tumors (Pujol et al., 1998; Batistatou et al., 2004; Leung et al., 2006; Bergner et al., 2007). In some of these cancers, including breast and glial tumors, ER β levels decline in close correlation with the development of less differentiated phenotype (Batistatou et al., 2004, 2006; Stefanou et al., 2004). These findings are in agreement with our results, which show high levels of nuclear ER β in well differentiated desmoplastic and neuroblastic medulloblastomas (Table 1). Note however that some of the well differentiated medulloblastomas, and classic medulloblastomas (Table 1), were either weakly positive or negative for ER β . Despite this, we have observed a strong correlation between the detection of nuclear ER β and nuclear IRS-1. Presently, we could only speculate why poorly differentiated medulloblastomas are losing both ER β and nuclear IRS-1. This may be associated with the possibility that interaction between nuclear IRS-1 and Rad51 could be toxic in quickly proliferating cells, since the function of Rad51 is critical for the repair of both replicative and induced DNA damage (Hoeijmakers, 2001). Some support for this interpretation came from our preliminary observation, that we were unable to select stable clones of medulloblastoma cells transfected with IRS-1 mutant containing artificial nuclear localization signal (NLS-IRS-1). In these preliminary experiments, we have observed a massive co-localization between endogenous Rad51 and NLS-IRS-1, which was associated with cell cycle arrest and apoptosis. On the other hand, mouse medulloblastoma cells, BsB8, derived from the cerebellar tumors of transgenic mice expressing JCV T-antigen, proliferate quickly despite of the presence of nuclear IRS-1 (Wang et al., 2001). There are two instances however, which could explain this potential discrepancy: (i) JCV T-antigen and Rad51 bind to the N-terminal domain of IRS-1, therefore the fraction of nuclear IRS-1, which is still bound to JCV T-antigen, can not interact with Rad51 (Lassak et al., 2002; Trojanek et al., 2003); and (ii) proliferating BsB8 cells in culture have a tendency of losing JCV T-antigen and nuclear IRS-1 (Krynska et al., 2000; Lassak et al., 2002). The situation is quite different when IRS-1 is translocated to the nucleus by ER β . In such a scenario, ER β binds to the C-terminal domain of IRS-1, and the IRS-1-ER β nuclear complex can still interfere with Rad51, possibly inhibiting the progression of the replication forks at the sites of damaged DNA. Therefore, it is feasible to speculate that in slowly proliferating cells although nuclear IRS-1 can still inhibit HRR, the cells have enough time to repair damaged DNA in G1 phase of the cell cycle, by utilizing less faithful non-homologous end joining (NHEJ) (Pierce and Jasin, 2001; Lundin et al., 2002). This on the other hand can be associated with the accumulation of additional mutations and

the selection of cells with growth advantage. Once cell proliferation accelerates, more replicative DNA damage can accumulate, and the presence of nuclear IRS-1 may become a limiting factor. This in turn may favor selection of cells in which nuclear IRS-1 or even total IRS-1 will be eliminated. This has been already demonstrated in some androgen-independent prostate cancer (Reiss et al., 2001), and some estrogen-independent breast cancer cells, which despite of growth and survival promoting function of IRS-1, lost its expression (Jackson et al., 1998; Dalmizrak et al., 2007).

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Fig. 1.

Immunohistological analysis of human medulloblastoma biopsies. Immunohistochemistry was performed using the avidin–biotin–peroxidase complex system. The sections of normal human cerebellum and two medulloblastoma biopsies were immunolabeled with anti-IRS-1 antibody (rabbit polyclonal, Upstate Biotechnology, Temecula, CA), and anti-ER β antibodies (rabbit polyclonal, Abcam, Cambridge, MA), and microscopic evaluation was performed under the original magnification of 1,000 $\times$.

Fig. 2. Immunohistofluorescent detection of IRS-1 and ER β . Double immunofluorescent labeling was performed with anti-ER β (green fluorescence) and anti-IRS-1 (red fluorescence) antibodies using medulloblastoma clinical sample #9 from Table 1. Image marked “Double labeling” represents superimposition of two images in which ER β and IRS-1 were detected. Note the presence of a large number of medulloblastoma nuclei in which ER β and IRS-1 are co-expressed in the nucleus (yellow fluorescence).

Fig. 3. Protein levels and subcellular localization of ERs and IRS-1 in medulloblastoma cell lines. Part A: Western blot analysis showing ERβ, ERα, and IRS-1 protein levels in three human (Daoy, D384, D425) and two mouse (Bs-1a and BsB8) medulloblastoma cell lines. Human breast cancer cell line, MCF7, was used as a positive control. Loading conditions are verified by re-probing the same blot with anti-Grb-2 antibody. Part B: Immunocytofluorescent evaluation of ERβ in D384 and BsB8 cells growing in the presence of 10% FBS. D384 cells collected by cytopsin on poly-D-lysine coated slides, and BsB8 monolayer cultures were both fixed and immunolabeled with anti-ERβ mouse monoclonal antibody (green fluorescence). The nuclei were counterstained with DAPI (blue fluorescence).

Fig. 4.

The activity of estrogen responsive elements (EREs). The ERE transcriptional activity was evaluated in D384, BsB8, and Daoy cells by dual-Firefly/Renilla luciferase reporter assay (Promega). The cells were transfected with 50 ng of pERE_{luc} plasmid (Morelli et al., 2004) using Amaxa nucleoporator according the manufacturer recommendations, and were cultured in the presence of 10% FBS for 24 h. Subsequently the culture medium was changed to SFM and cells were stimulated with 100 mM estradiol (E2), 10 μ M ICI 182,780 (ICI), or were left without stimulation (control). The measurements of luciferase activity were taken 6 h after stimulation. Results represent an average of three separate experiments in duplicate ($n=6$).

*Indicates values significantly different from the control ($P \leq 0.05$).

Fig. 5.

Evaluation of IRS-1-ERβ interaction. Part A: The interaction between ERβ and IRS-1 evaluated by Immunoprecipitation/Western blot analysis (IP/W). Total proteins (500 μg/ml) were IP with anti-ERβ antibody, and the resulting nitrocellulose filters were probed with anti-IRS-1 and anti-ERβ antibodies, respectively. In control conditions, 50 μg aliquots of the protein extract were loaded without IP (No IP)—positive control; or IP was carried out without anti-ERβ antibody (No Ab)—negative control. Parts B and C: Double immunolabeling with anti-ERβ (green) and anti-IRS-1 (red) antibodies (B), and with anti-IRS-1 (green) and anti-histone-γH2AX antibodies (red) (C). The D384 and Daoy cells were cultured in 10% FBS and treated with cisplatin (1 μg/ml for 6 h). Images labeled “Overlap” represents superimposition of three images, which consist of either ERβ and IRS-1 (B); or IRS-1 and γH2AX immunolabelings (C), and DAPI labeled DNA (blue). Digital sections (2 μm) from D384 and Daoy cells were collected by the inverted fluorescent microscope equipped with motorized Z-axis and the deconvolution software (SlideBook4). Note possible co-localization (yellow fluorescent foci) between IRS-1 and ERβ detected in the nuclei of exponentially growing Daoy and D384 cells, or between IRS-1 and histone γH2AX detected in the nuclei of exponentially growing Daoy cells.

Fig. 6.

Nuclear interaction between IRS-1 and Rad51. Part A: Double immunolabeling with anti-Rad51 (green) and anti-IRS-1 (red) antibodies was applied to exponentially growing Daoy cells at 6 h after the treatment with cisplatin (1 μg/ml). The image marked “Overlap” represents superimposition of three images in which IRS-1, Rad51, and DAPI labeling were applied. A digital section from a single Daoy cell was obtained by inverted fluorescent microscope equipped with motorized Z-axis and the deconvolution software (SlideBook4). Part B: Quantification of the co-localization between IRS-1 and Rad51 was calculated from the entire volume of the nucleus by utilizing Slide Book 4 software (Intelligent Imaging Innovations, Denver CO), according to the manufacturer instructions for mask operations. Data represent average values obtained from the evaluation of 10 randomly selected cells in three independent experiments (n =30). Part C: Western blot analysis with proteins isolated from nuclear (N) and cytosolic (C) fractions of exponentially growing Daoy cells in the presence (FBS +ICI) or absence (FBS) of ERαβ/antagonist, ICI 182,780. The blot was probed first with anti-IRS-1 antibody and was re-probed with anti-ERβ antibody (predominant nuclear localization), and with anti-Grb-2 antibody (cytosolic marker) to evaluate potential contamination of the nuclear fraction with cytosolic fraction. Note that cells treated with ICI 182,780 are characterized by a much lower level of nuclear IRS-1. Part D: The interaction between Rad51 and IRS-1 evaluated by IP/Western. Total proteins (500 μg/ml) were IP with anti-Rad51 antibody, and

resulting nitrocellulose filters were probed with anti-IRS-1 and anti-Rad51 antibodies. In control conditions, 50 µg aliquots of the protein extract were loaded without IP (Western)—positive control; or IP was carried out without anti-Rad51 antibody (No Rad51 Ab)—negative control. Note that there is significantly less IRS-1, which co-precipitates with anti-Rad51 antibody in Daoy cells pretreated with ICI 182,780 (n =3). * Indicates value significantly different from FBS. ** Indicate value significantly different from E2 ($P \leq 0.05$).

Fig. 7.

ERβ-IRS-1 interaction affects HRR. Part A: GST-pull down assay was performed to determine which portion of the IRS-1 molecule binds ERβ. A collection of GST-IRS-1 overlapping truncation mutants (Lassak et al., 2002) and nuclear extracts from Daoy cells were utilized to pull down ERβ. Following pull down reaction one of the five IRS-1 truncation mutants, GST-IRS-1 (931-1233), bound ERβ. All other mutants and GST alone were negative. Part B: Schematic illustration of the pcDNA3/IRS-1-931-1233 —the expression vector in which the 931-1233 C-terminal fragment of IRS-1 was cloned in frame with Myc tag under the control of CMV promoter. Part C: Western blot analysis with protein extracts from Daoy cells expressing the 931-1233/IRS-1 mutant. The cells were transfected in transient (Amaxa nucleoporator) with the pcDNA3/IRS-1-931-1233 or with pcDNA3 empty vector (EV). Proteins were isolated at 24, 48, and 72 h after transfection and the resulting blots were probed with anti-IRS-1 rabbit polyclonal antibody, which recognizes C-terminal domain of IRS-1 (UBI, Lake Placid, NY). The cells transfected with 931-1233/IRS-1 mutant, express endogenous IRS-1 (160 kDa band) and expected 30 kDa band (931-1233 IRS-1). Two lower bands were detected as well. These additional bands may represent products of restricted proteolysis from the N-terminal end of this fusion protein, since the same blot striped and re-probed with anti myc-tag antibody, demonstrated the same pattern and intensity of the bands. Note that myc-tag was cloned at the C-terminus of 931-1233 IRS-1. Part D: Homologous

recombination directed DNA repair (HRR) was evaluated by the DRGFP reporter system. Daoy/DRGFP cells growing in 10%FBS (control) were additionally treated with 17 β -estradiol (E2)(100mM), ICI 182,780(10 μ M) or were transfected with the pcDNA3-IRS-1-931–1233 expression vector. To evaluate HRR, the cells were transfected in transient (Amaxa) with I-*Sce*I and mito-red expression vectors, and the reconstitution of GFP function was analyzed under fluorescent microscope and quantified by flow-cytometry. The results were collected from three separate experiments, in duplicate (n =6). Note a significant increase in HRR in Daoy cells treated either with ICI182, 780 or transfected with the 931–1233-IRS-1 mutant. * Indicates values significantly different from control. ** Indicate values significantly different from EV ($P \leq 0.05$). The inset in Figure 6D illustrates an example of HRR-mediated reconstitution of GFP function in Daoy/DRGFP cells treated with ICI 182,780.

TABLE 1

Diagnosis of the tumors is based on the World Health Organization classification of tumors of the CNS

No.	Diagnosis	IRS-1	Estrogen receptor β
1	Medulloblastoma—neuroblastic	++ Cytoplasmic	—
2	Medulloblastoma—classic	+ Cytoplasmic	—
3	Medulloblastoma—classic	+ Cytoplasmic	—
4	Medulloblastoma—classic	+ Nuclear	+ Nuclear
5	Medulloblastoma—neuroblastic	++ Cytoplasmic/+ nuclear	—
6	Medulloblastoma—neuroblastic	++ Nuclear	+ Nuclear
8	Medulloblastoma—classic	—	—
9	Medulloblastoma—neuroblastic	++ Nuclear	+++ Nuclear
10	Medulloblastoma—classic	++ Cytoplasmic/+ nuclear	—
11	Medulloblastoma—classic	—	—
12	Medulloblastoma—desmoplastic	+ Nuclear	++ Nuclear
13	Medulloblastoma—classic	—	—
14	Medulloblastoma—desmoplastic	+ Nuclear	+ Nuclear
15	Medulloblastoma—neuroblastic	++ Cytoplasmic/++ nuclear	++ Nuclear
16	Medulloblastoma—neuroblastic	+ Cytoplasmic	—
17	Medulloblastoma—neuroblastic	++ Cytoplasmic	—
18	Medulloblastoma—classic	—	—
19	Medulloblastoma—classic	+ Cytoplasmic/+ nuclear	—
20	Medulloblastoma—classic	+ Cytoplasmic	—

+ , Weak immunoreactivity;

++ , moderate immunoreactivity;

+++ , robust immunoreactivity.