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Biochemical and Biophysical Research Communications 311 (2003) 174–178
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Roles of the insulin-like growth factor I receptor C-terminus in cellular radioresistance

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Received 23 September 2003

Abstract

Available evidence suggests that insulin-like growth factor I receptor (IGF-IR) expression leads to increased cellular radioresistance. The most direct explanation of these findings predicts that IGF-IR is the source of survival signals in resistant cells. Mutational analysis revealed that protein truncated at amino acid 1245 in the C-terminus retained the ability of IGF-IR to confer radioresistance whereas point mutations at both Tyr-1250 and Tyr-1251 abrogated this effect using IGF-IR-deficient mouse embryo fibroblasts (R−) as a recipient. In cells expressing the latter mutant receptors, both phosphatidylinositol-3′ kinase (PI3-K) and mitogen-activated protein kinase (MAPK) signaling pathways remained intact, and addition of exogenous IGF-I could not change the radiosensitivity of these cells. Further analysis indicated that the abrogation of radioresistance required the presence of His-1293 and Lys-1294. These results suggest a novel regulatory role of the C-terminus of IGF-IR in mediating cellular radioresistance that may be independent of survival signals transmitted through this receptor.

Keywords: Insulin-like growth factor I receptor; Radioresistance; Survival signal; Mutational analysis; Phosphatidylinositol-3′ kinase; Mitogen-activated protein kinase

Activation of insulin-like growth factor I receptor (IGF-IR) plays an important role in preventing cell death following a variety of stimuli including ionizing irradiation [1–5]. IGF-IR is thought to inhibit cell death through the activation of three downstream signaling pathways: phosphatidylinositol-3 kinase (PI3-K), mitogen-activated protein kinase (MAPK), and 14-3-3 proteins [6,7]. It is thus possible that IGF-IR-mediated radioresistance is caused by the enhanced stimulation of these signaling pathways. Indeed, we have reported that these three pathways play partially overlapping roles in radioresistance using mouse embryo fibroblasts deficient in IGF-IR [8]. In the present study, using the same model system, we show evidence that IGF-IR-mediated radioresistance could be abrogated by certain mutations in the C-terminus while the activation of downstream survival signaling pathways are still maintained.

Materials and methods

Materials. Antibodies against IGF-IR α- and β-subunits, ERK2, goat IgG conjugated with horseradish peroxidase (HRP), rabbit IgG-HRP, and protein A/G PLUS-agarose were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine antibody (PY20) was purchased from Transduction Laboratory (Lexington, KY) and anti-PKB/AKT pSer(473) antibody was purchased from Biosource (Camarillo, CA). Anti-ACTIVE MAPK antibody was purchased from Promega (Madison, WI), and anti-β actin antibody was purchased from Chemicon International (Temecula, CA). The ECL Western blotting analysis system was purchased from Amersham–Pharmacia Biotech (Arlington Heights, IL). Recombinant human IGF-I was purchased from Invitrogen (Gaithersburg, MD).

Cell lines and culture conditions. Mouse embryo fibroblasts deficient in IGF-IR (R−) were used as recipients of mutant human IGF-IRs and maintained as described previously [5].

Plasmid construction and transfection. Wild type (WT), Δ1310, Δ1293, Δ1245, Y50-51F (tyrosines 1250 and 1251 mutated to phenylalanines), and H93F-K94L (histidine 1293 and lysine 1294, respectively, mutated to phenylalanine and leucine) receptor mutants were derived from human IGF-IR cDNA [9] as described previously [10,11]. For Y50-51F/Δ1307, Y50-51F/Δ1292, and Y50-51F/Δ1279 double mutants, PCR-assisted in vitro mutagenesis was performed using

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doi:10.1016/j.bbrc.2003.09.195

pBluescript Y1250F/Y1251F as a template [10]. Primers used were as follows: 5′-primer, ACAGTGAACGAGGCCGCAAG; 3′-primer, 5′-GGATCCTCAGGCCGAGGGGTCCAGG (Y50-51F/Δ1279); 5′-GG ATCCTCATCCTGAGTGTCTGTCG (Y50-51F/Δ1292); and 5′-GG ATCCTCAGCGGAGGACCAGCAC (Y50-51F/Δ1307). All three 3′-primers were designed to create a *Bam*HI site located 6 base pairs past the stop codon of the IGF-IR cDNA. The 5′-primer was designed to locate beyond the unique *Hin*dIII site. The PCR products were subcloned into a PCR-Blunt vector (Invitrogen). A WT *Hin*dIII–*Bam*HI fragment of a pBluescript SK *Sal–Bam* IGF-IR (a pBluescript SK phagemid containing a *Sal*I–*Bam*HI fragment of the full IGF-IR cDNA) was replaced by the mutant *Hin*dIII–*Bam*HI fragment from the PCR-Blunt vector containing the PCR products, and the *Xho*I–*Not*I fragment of pBluescript SK Y1250F/Y1251F including the corresponding fragment of truncated IGF-IR cDNA. For the Y50-51F/H93F-K94L mutant, the initial PCR was performed using pBluescript SK Y1250F/Y1251F as a template and primers as follows: 5′-primer, 5′-AGGGCCGGGCCCGTTCTCGGCCCAGGAATCCTG; 3′-primer (mutagenic primer), 5′-CAGGATTCCTGGCCGAGAACGGG CCCGGCCCT. The 3′-primer was designed to create an *Apa*I site with silent mutations. The second PCR was then performed using the same template and primers as follows: 5′-primer, the first PCR products; 3′-primer, 5′-AATTAACCCTCACTAAAGGG (T3 primer in the pBluescript SK). The products were subcloned into a PCR-Blunt vector and replaced by the mutant *Hin*dIII–*Bam*HI fragment into pBluescript Y1250F/Y1251F. Finally, the *Xho*I–*Not*I fragment of pBPV IGF-IR was replaced with the corresponding fragment containing the above-described mutations. All the mutations created by PCR were confirmed by the dideoxy-sequencing method. Expression plasmids containing WT or mutant IGF-IR cDNA were stably transfected into R− cells with a pPDV6+ plasmid by calcium phosphate precipitation as described previously [10]. Mixed populations or clones were selected after puromycin treatment. At least two independent clones were isolated for each mutant with similar receptor expression levels and used for examining radiosensitivity.

Western blotting. Western blotting was performed as described previously [8] using the ECL Western blotting analysis system. For immunoprecipitation, IGF-I-unstimulated or -stimualated cells were lysed and IGF-IRβ was immunoprecipitated with anti-IGF-IRβ antibody bound to protein A/G PLUS-agarose. Immunoprecipitates were washed and subjected to Western blotting. Autophosphorylation of IGF-IR was visualized using anti-phosphotyrosine antibody (PY20) as described above.

Colony-forming assay. Radiosensitivity was evaluated by dose–survival curves as determined by a colony-forming assay as described previously [5]. After 7–10 days of incubation, cells were fixed and stained with crystal violet. Colonies containing more than 50 cells were counted; the surviving fractions were then determined.

γ-Irradiation. γ-Irradiation was performed using ^{60}Co γ-ray therapeutic machine at a dose rate of 1.4 Gy/min.

Results and discussion

We previously reported that truncation of the C-terminal region of human IGF-IR at residue 1245 in human IGF-IR (Δ1245) did not effect IGF-IR-mediated clonogenic radioresistance in mouse embryo fibroblasts deficient in endogenous IGF-IR (R−). Mutational analysis revealed that signals from either of the two different sites, the tyrosine residue 950 or the C-terminus, presumably quartet serine residues 1280–1283, are sufficient for these effects. Indeed, both domains must be mutated to abrogate radioresistance [8]. Consistent with these results, cells with two additional deletion constructs, Δ1310 and Δ1293 (Fig. 1A), with expression levels comparable to WT cells (Fig. 1B), exhibited the same degree of clonogenic radioresistance as WT cells (Fig. 1C). Surprisingly, point mutations at tyrosine residues 1250 and 1251 (tyrosines 1250 and 1251 mutated to phenylalanines) (Y50-51F) completely abrogated IGF-IR-mediated radioresistance and cells expressing the mutant IGF-IR were as radiosensitive as R− cells (Fig. 1C).

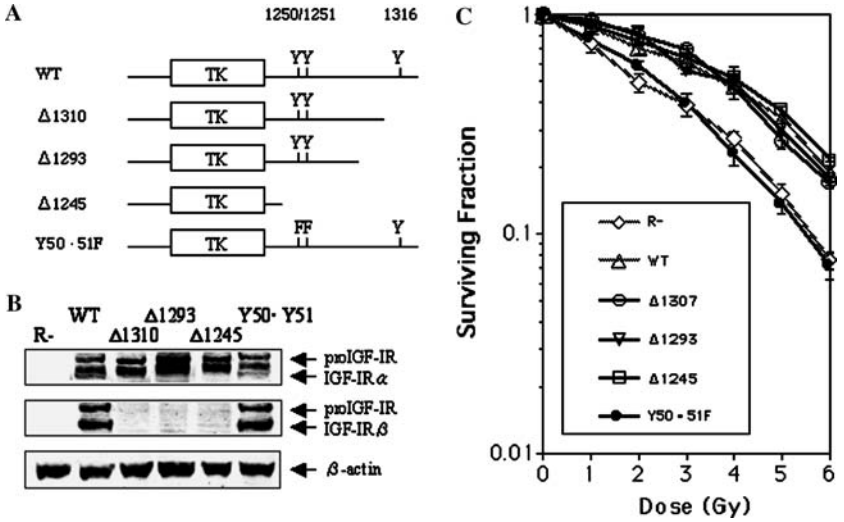

Fig. 1. Mutational analysis on clonogenic radiosensitivity in varying C-terminal mutants of IGF-IR. (A) Schematic presentation of the IGF-IR β-subunit with mutations at its C-terminus. Y, tyrosine; and F, phenylalanine. (B) Expression levels of mutant IGF-IRs. Cell lysates were prepared for Western blotting as described under Materials and methods. The anti-IGF-IR β antibody used in this study recognizes the C-terminal region of the β-subunit. (C) Dose–survival curves of the C-terminal mutants following irradiation. Results were presented as means ± SD of triplicate determinants. Additional independently derived clones showed similar results.

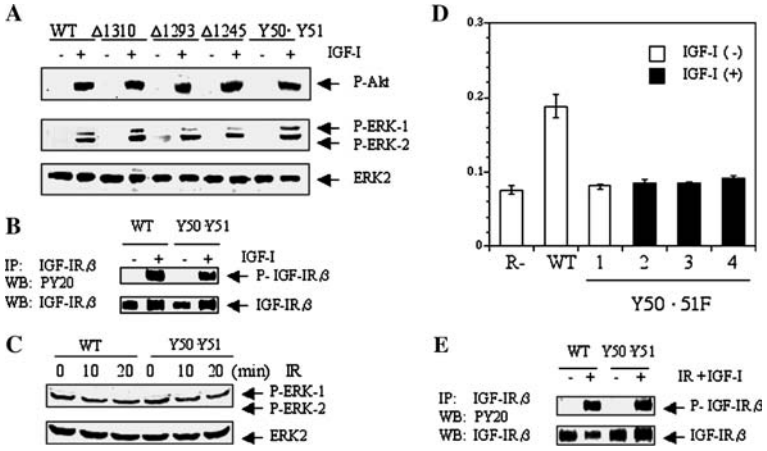

Fig. 2. Characterization of survival signals originating from IGF-IR with mutations at residues 1250 and 1251. (A) Activation of PI3-K and MAPK pathways in different C-terminal mutants upon IGF-I stimulation. Serum-deprived cells were unstimulated or stimulated with 50 ng/ml of IGF-I for 10 min and prepared for Western blotting as described under Materials and methods. (B) Autophosphorylation of IGF-IR in WT and Y50-51F mutant upon IGF-I stimulation. Serum-deprived cells were unstimulated or stimulated with 50 ng/ml IGF-I and cell lysates were immunoprecipitated with anti-IGF-IRβ antibody (IP: IGF-IRβ) as described under Materials and methods. Immunoprecipitates were subjected to Western blotting and probed with PY20 or anti-IGF-IR antibody. (C) Effect of irradiation on MAPK activation. Cells in growth medium were lysed at the indicated times after 6 Gy of irradiation and activated ERK was monitored by Western blotting. IR: ionizing irradiation. (D) Effect of exogenously added IGF-I on clonogenic radiosensitivity in the Y50-51F mutant. Cells were unstimulated (1) or stimulated with IGF-I (50 ng/ml) 10 min before irradiation (6 Gy) (2), immediately after irradiation (3) or 10 min after irradiation (4). IGF-I-treated or untreated cells at the different timings were allowed to form colonies and survival fractions were determined. (E) Effect of irradiation on IGF-I-induced autophosphorylation in WT and Y50-51F mutant. Cells irradiated with 6 Gy were incubated for 10 min and unstimulated or stimulated with 50 ng/ml IGF-I for 10 min. Cell lysates were immunoprecipitated with anti-IGF-IRβ antibody (IP: IGF-IRβ) and autophosphorylation was detected as described above.

The inability of the Y50-51F mutant to confer radioresistance to R− cells could be explained if this mutation disrupted the ability of IGF-IR to transmit survival signals. Contrary to this, all mutants examined were able to similarly stimulate the PI3-K/Akt and MEK/ERK pathways upon IGF-I stimulation (Fig. 2A). Studies employing PI3-K inhibitors have demonstrated that activation of Akt is absolutely dependent on IGF-I-induced PI3-K activity [8]. Given the differences between WT and Y50-51F cells, these cell lines were selected to further examine IGF-IR-mediated signaling events. Levels of IGF-IR autophosphorylation upon IGF-I stimulation were very similar between the two cell lines (Fig. 2B). Since activation of the MEK/ERK, but not PI3-K pathway, could singly induce clonogenic radioresistance in R− cells by our previous study [8], activation of the MEK/ERK pathway was examined following irradiation. Irradiation (6 Gy) did not reproducibly induce significant activation of ERK in either cell line under the conditions tested (Fig. 2C). Very low levels of IGF-IR stimulation are enough to induce IGF-IR-mediated radioresistance [8], thus a subtle reduction in the signaling capacity of the Y50-51F mutant that was not detectable in the assay conditions used could be responsible for the altered radioresistance seen in these cells. Treatment of cells with high levels of IGF-I should overcome any subtle differences, but increased survival was not observed with any IGF-I treatment at different time-points during irradiation (Fig. 2D). While unlikely, it is possible that irradiation could render the mutant IGF-IR incapable of transmitting survival signals. Following 6 Gy of irradiation, however, the mutant IGF-IR responded to IGF-I stimulation with similar amounts of autophosphorylation as seen in WT cells (Fig. 2E). Activation of ERK was also similarly observed (data not shown). Taken together, these results suggest that a novel mechanism may exist whereby IGF-IR-mediated radioresistance does not occur despite the presence of sufficient survival signal.

Further C-terminal deletion analysis using the Y50-51F mutant was undertaken. The structures of mutant receptors and their expression levels are shown in Figs. 3A and B. Activation levels of ERK and Akt were similar among the mutants following IGF-I stimulation (data not shown). Cells with truncation of IGF-IR at residue 1307 in the presence of mutation at residues 1250 and 1251 (Y50-51F/Δ1307) exhibited a radiosensitive phenotype similar to the Y50-51F cells. Interestingly, Y50-51F/Δ1292 and Y50-51F/Δ1279 mutant constructs conferred similar radioresistance as WT IGF-IR in transfected R− cells (Fig. 3C, left panel). These findings indicate that abrogation of radioresistance by the mutations at residues 1250 and 1251 requires the presence of at least residues 1293–1307. Previous reports by Liu et al. [12] and Hongo et al. [13] showed that expression of a membrane-targeted IGF-IR C-terminal region

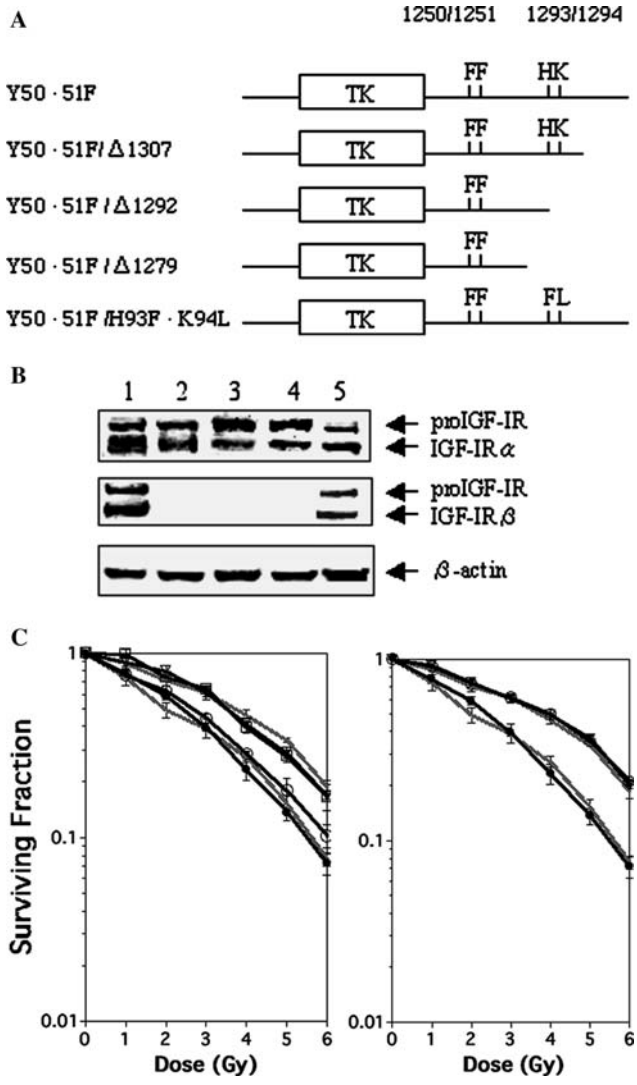

Fig. 3. Mutational analysis on clonogenic radiosensitivity in varying C-terminal mutants with mutations at residues 1250 and 1251. (A) Schematic presentation of the IGF-IR β-subunit with mutations at its C-terminus. F, phenylalanine; H, histidine; K, lysine; and L, leucine. (B) Expression levels of mutant IGF-IRs. Western blotting was performed as described in Fig. 1. Lane 1, Y50-51F; lane 2, Y50-51F/Δ1307; lane 3, Y50-51F/Δ1292; lane 4, Y50-51F/Δ1279; and lane 5, Y50-51F/H93F-K94L. (C) Dose–survival curves in varying deletion mutants with mutations at residues 1250 and 1251 (left panel). Gray line, WT and R−; circle, Y50-51F/Δ1307; inverted triangle, Y50-51F/Δ1292; square, Y50-51F/1279; and closed circle, Y50-51F. Dose–survival curves in two independently established clones expressing mutant IGF-IR with mutations at residues 1250/1251 and 1293/1294 (right panel). Gray line, WT and R−; circle, Y50-51F/H93F-K94L #1; inverted triangle, Y50-51F/H93F-K94L #2; and closed circle, Y50-51F. Results are presented as means ± SD of triplicate determinants.

induces cytotoxicity, and the effect was completely abrogated by mutations at residues His-1293 and Lys-1294. These residues are included in the region in question. We therefore constructed mutant receptors with mutations at residues 1250/1251 and 1293/1294 (histidine 1293 and lysine 1294 mutated, respectively, to phenylalanine and leucine) (H93F-K94L), and examined the ability of the receptor to confer clonogenic radioresistance. Mutation of all four residues rendered R− cells as radioresistant as transfectants with WT constructs as shown by two independent clones #1 and #2 (Fig. 3C, right panel). Mutations of residues 1293 and 1294 alone did not affect clonogenic radioresistance, yielding the same survival curves as WT cells (data not shown).

Intrinsic radiosensitivity is an important factor that influences local control or survival following radiotherapy [14]. Overexpression of epidermal growth factor receptor (EGFR) is often observed in a variety of tumors, and some reports describe its association with radioresistance through the activation of downstream survival signaling pathways [15,16]. IGF-IR is also overexpressed in some tumors including breast, colon, prostate, and brain, and poor clinical results have been reported following radiotherapy in IGF-IR-overexpressing breast cancers [3,17,18]. Several lines of evidence indicate that IGF-IR induces cellular radioresistance [3–5] and we recently reported that downstream survival signaling pathways, MAPK, PI3-K, and 14-3-3 proteins, are associated with IGF-IR-mediated clonogenic radioresistance in mouse embryo fibroblasts in a partially overlapping manner [8]. Using this model system, we found unexpected functions of the IGF-IR C-terminus in modulating clonogenic radioresistance in this study; point mutations at tyrosine residues 1250 and 1251, but not deletion of a larger portion of the C-terminal region, abrogated clonogenic radioresistance. Interestingly, both mutant receptors possessed abilities to activate downstream signaling pathways including PI3-K and MAPK to the same extent.

Previous reports have demonstrated that expression of a membrane-targeted C-terminal construct of IGF-IR induces massive cell death. This effect was not inhibited by overexpression of WT IGF-IR, indicating that the pro-death signals do not compete with IGF-IR-mediated survival signals. Furthermore, residues His-1293 and Lys-1294 were identified as being essential for these effects [12,13]. This finding is especially of interest given that coexistence of the residues 1293 and 1294 was required for the abrogation of radioresistance by mutation of residues 1250 and 1251. It raises the possibility that the hypothesized death promoting signals may arise from the C-terminus following irradiation but that these signals are normally inhibited by tyrosine residues 1250 and 1251. The molecular mechanism related to the pro-death signals from the C-terminus and its detailed regulation in the whole IGF-IR molecule still remain unclear. The elucidation of such mechanisms may prove invaluable in devising potential therapeutic interventions to increase the radiosensitivity of some solid tumors.

Acknowledgments

This work was supported in part by Grant-in-aid for Scientific Research on Priority Areas (13218045) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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