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Synergistic action of R-Ras and IGF-1 on Bcl-xL expression and caspase-3 inhibition in BaF3 cells: R-Ras and IGF-1 control distinct anti-apoptotic kinase pathways

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Abstract R-Ras and insulin-like growth factor-1 (IGF-1) synergistically inhibit apoptosis of BaF3 cells upon interleukin-3 deprivation. To characterize the mechanism of this synergistic inhibition, we examined the effect of R-Ras and IGF-1 on several apoptosis-related proteins. Extracellular signal-regulated kinase (ERK) was activated by IGF-1, but not by R-Ras. In contrast, Akt was activated strongly by R-Ras, but weakly by IGF-1. It was also found that R-Ras and IGF-1 cooperatively induced Bcl-xL expression and inhibited caspase-3 activation.

Key words: R-Ras; Apoptosis; Insulin-like growth factor-1; Akt; Mitogen-activated protein kinase; Bcl-x

1. Introduction

R-Ras is a low-molecular-weight GTP-binding protein which shares about 55% homology with H-Ras oncoprotein [1]. Several possible target molecules of R-Ras have been reported, such as Raf [2,3], phosphatidylinositol 3-kinase (PI3K) [4] and RalGDS [5]. Since these molecules are also known to be direct targets of H-Ras, R-Ras would appear to share some signaling pathway with H-Ras. However, R-Ras was initially reported to possess no oncogenic potential on Rat1 fibroblast [6]. Although it was later found that R-Ras can cause malignant transformation of another fibroblast, NIH 3T3 cells, R-Ras does not induce morphological change [7]. Besides transformation, R-Ras has been reported to modulate cell adhesion to extracellular matrix proteins through integrins [8] and to be involved in regulation of apoptosis [9,10].

Apoptosis is a physiological process that eliminates unnecessary or injured cells. Recent studies have revealed that Bcl-2 family proteins, caspases, the PI3K-Akt (also known as protein kinase B or Rac kinase) pathway, and the Raf-ERK (also known as MAPK) pathway play crucial roles in apoptosis. Bcl-2, which was originally identified through the study of the chromosome translocation present in human B-cell follicular lymphomas [11], is a mammalian homologue of Ced-9, an anti-apoptotic protein in *Caenorhabditis elegans* [12]. A number of proteins that are structurally related to Bcl-2 have been identified and classified into two groups: anti-apoptotic proteins (Bcl-2, Bcl-xL, and Bcl-w) and pro-apoptotic proteins (Bax, Bad, and Bcl-xS). Although it is not clear how anti-apoptotic Bcl-2 family proteins induce cell survival, it has been suggested that they somehow inhibit activation of caspases, cysteine proteases which cleave numerous target proteins to induce apoptosis [13,14]. PI3K is activated by several growth factors and cytokines that promote survival, and is required for their anti-apoptotic activities [15]. Activated PI3K converts PIP_2 to PIP_3 by phosphorylation. PIP_3 then binds to Akt and stimulates its kinase activity, which leads to suppression of apoptosis [15–17]. The Raf-ERK pathway is also activated by several survival factors [18] and has been reported to regulate cell survival. For instance, ERK activity is required for survival of PC-12 cells on deprivation of nerve growth factor [19].

In our previous work, we found that R-Ras and serum synergistically inhibit apoptosis induced by interleukin (IL)-3 deprivation in BaF3, a pro-B cell line whose proliferation and survival are strictly dependent on IL-3 [10]. Furthermore, we identified insulin-like growth factor-1 (IGF-1) as an essential factor in serum for R-Ras-induced suppression of cell death. In addition, studies using inhibitors suggested that the PI3K-Akt pathway and the Raf-ERK pathway are involved in this suppression. However, it remained unknown how R-Ras and IGF-1 cooperatively support survival of BaF3. In this paper, we investigated the effects of R-Ras and IGF-1 on the activities of Akt, ERK and caspase, and the expression of anti-apoptotic Bcl-2 family protein.

2. Materials and methods

2.1. Materials

Plasmid pCMV5-R-Ras^{Q87L} was generated from pUC12-R-*ras*, a generous gift from D.V. Goeddel (Genentech, South San Francisco, CA, USA), as described previously [10]. It should be noted here that we found that the sequence of mouse R-*ras* cDNA on pUC12-R-*ras* differs from the published sequence (Lowe et al. [1]) at Tyr^{193} (TAC) → His (CAC). Complementary DNA of Akt was kindly provided by U. Kikkawa (Kobe Univ., Japan) and Myc-tagged Akt was subcloned into the *Hin*dIII and *Xba*I sites of pCMV5 [20] to obtain pCMV5-Myc-Akt [21]. Mouse IL-3 was generously provided by A. Miyajima and S. Menon (DNAX Research Institute, Palo Alto, CA, USA). Human IGF-1 was purchased from Life Technologies (Rockville, MD, USA); anti-Myc antibody (9E10) from Boehringer Mannheim (Indianapolis, IN, USA); anti-ERK2 antibody (sc-154) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-R-Ras antibody (#15626E) from Pharmingen (San Diego, CA, USA); and anti-Bcl-x antibody (B22620) from Transduction Laboratories (Lexington, KY, USA).

2.2. Cell culture

A BaF3 transfectant, Lh9 [10], was established using the LacSwitch inducible mammalian expression system (Stratagene, La Jolla, CA, USA). BaF3 cells were maintained in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum and mouse IL-3. For Lh9 cells, G418 (1 mg/ml) and hygromycin (1 mg/ml) were included in the culture medium. The serum-free medium was prepared as previously described [22].

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2.3. Kinase assay

For ERK assay, Lh9 cells were pre-cultured for 12 h in the presence or absence of IPTG (5 mM). After 3 h starvation in RPMI 1640 containing 1 mg/ml bovine serum albumin (BSA) with or without IPTG, cells were stimulated with IGF-1. From cell lysates, endogenous ERK was immunoprecipitated with anti-ERK2 antibody and its kinase activity was measured using myelin basic protein (MBP) as a substrate according to the procedure previously described [10]. For Akt assay, 2×10^7 BaF3 cells were transfected with 30 μg of pCMV5-Myc-Akt together with 20 μg of either pCMV5 or pCMV5-R-Ras^{Q87L} by electroporation (300 V, 960 μF) and cultured for 1 day. Then cells were starved for 3 h in RPMI 1640 containing BSA (1 mg/ml). After stimulation with IGF-1, cells were lysed and Myc-Akt was immunoprecipitated with 0.5 μl of anti-Myc antibody from 150 μg of lysate. After washing, the precipitate was incubated at 30°C for 20 min with 2 μg of histone 2B in the presence of 20 μM [γ-^{32}P]ATP (620 TBq/mol). The reaction was stopped by boiling in the sample buffer for SDS-PAGE. The samples were resolved by SDS-PAGE, and the radioactivity incorporated into histone 2B was quantitated by image analyzer (Fuji BAS2000).

2.4. mRNA and protein analysis of Bcl-x

For mRNA analysis, Lh9 cells (1×10^6) were pre-cultured for 12 h in the presence or absence of IPTG (5 mM). After 3 h starvation with the serum-free medium, cells were incubated for 3 h with IGF-1 or IL-3 in the presence or absence of IPTG. Total RNA was then extracted utilizing TRIZOL reagent (Life Technologies) and RT-PCR was performed by RT-PCR kit (Takara, Japan) according to the manufacturers' instructions. Briefly, total RNA (0.1 μg) was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase and random primers (9-mer). Then, *bcl*-x mRNA was amplified using the following primers: 5′-primer: 5′-ATGTCTCAGAGCAACCGGGAG-3′; 3′-primer: 5′-TCACTTCCGACTGAAGAGTGAGC-3′.

Amplified *bcl*-x mRNA was subjected to agarose gel electrophoresis (1.2% (w/v) agarose). For protein analysis, after 12 h culture in the presence or absence of IPTG (5 mM), Lh9 cells were transferred to the serum-free medium, and incubated for 24 h either in the presence or absence of IGF-1 and/or IPTG. Cells were then lysed, and a portion of lysate (25 μg of protein) was subjected to 10% SDS-PAGE, followed by immunoblot analysis using anti-Bcl-x antibody.

2.5. Caspase assay

After 12 h culture in the presence or absence of IPTG (5 mM), Lh9 cells (1×10^6) were incubated for 24 h with or without IGF-1 either in the presence or absence of IPTG. Cells were then solubilized in 100 μl of buffer (10 mM HEPES-NaOH, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT) and 10 μg of lysate was incubated with 40 μM DEVD-MCA (Ac-Asp-Glu-Val-Asp-α-(4-methyl-coumaryl-7-amide)) (Peptide Institute, Osaka, Japan) for 1 h at 37°C in 200 μl of buffer (10 mM HEPES-NaOH, pH 7.4, 2 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT, 50 mM NaCl). The reaction was stopped by addition of 200 μl of 0.2 M glycine-HCl (pH 2.8). Fluorescence of cleaved peptides was measured by F-2000 (Hitachi, Japan).

3. Results

3.1. Effects of R-Ras and IGF-1 on ERK and Akt activities in BaF3 cells

First, we examined the effect of IGF-1 stimulation and expression of an activated mutant of R-Ras (R-Ras^{Q87L}) on ERK activity in BaF3 cells. For this, we utilized a BaF3 transfectant, Lh9, which inducibly expresses R-Ras^{Q87L} in the presence of isopropyl β-D-thiogalactopyranoside (IPTG) [10]. Lh9 cells were pre-cultured in the culture medium either in the presence or absence of IPTG, and then deprived of IL-3 and serum for 3 h. After harvest, cells were lysed and the activity of endogenous ERK was measured by in vitro kinase assay. As shown in Fig. 1, IGF-1 activated ERK at a concentration of 100 ng/ml. On the other hand, the expression of R-Ras^{Q87L} failed to stimulate ERK activity.

We next analyzed the effect of R-Ras and IGF-1 on activity of Akt, another important kinase for survival. Myc-tagged Akt (Myc-Akt) was transiently expressed together with or without R-Ras^{Q87L} in BaF3 cells. Ectopically expressed Myc-Akt was then immunoprecipitated and in vitro kinase assay was performed using histone 2B as a substrate. Kinase activity of Akt was dramatically increased by expression of R-Ras^{Q87L} (Fig. 2). In contrast, stimulation by IGF-1 alone induced weak activation of Akt.

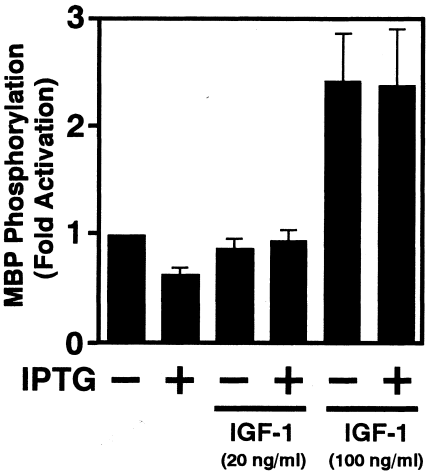

Fig. 1. Effect of R-Ras and IGF-1 on ERK activity. After 3 h starvation, Lh9 cells were incubated for 10 min at 25°C with IGF-1 (20 or 100 ng/ml). The values are expressed as the mean ± S.E. of three separate experiments.

3.2. Cooperative regulation of Bcl-xL expression by R-Ras and IGF-1

Since the expression level of Bcl-xL correlates well with survival efficiency of BaF3 cells [23,24], we studied whether R-Ras and IGF-1 affect the expression of Bcl-xL. After 3 h deprivation of IL-3 and serum, Lh9 cells were cultured further for 3 h in the presence or absence of IGF-1 and/or IPTG, and the mRNA of *bcl*-xL was amplified from total RNA by RT-

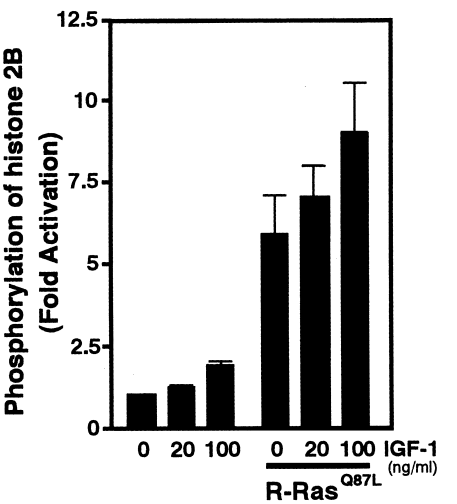

Fig. 2. Effect of R-Ras and IGF-1 on Akt activity. After 3 h starvation, transfected BaF3 cells were stimulated for 10 min at 25°C with the indicated concentration of IGF-1. Data are presented as the mean ± S.E. of three separate studies.

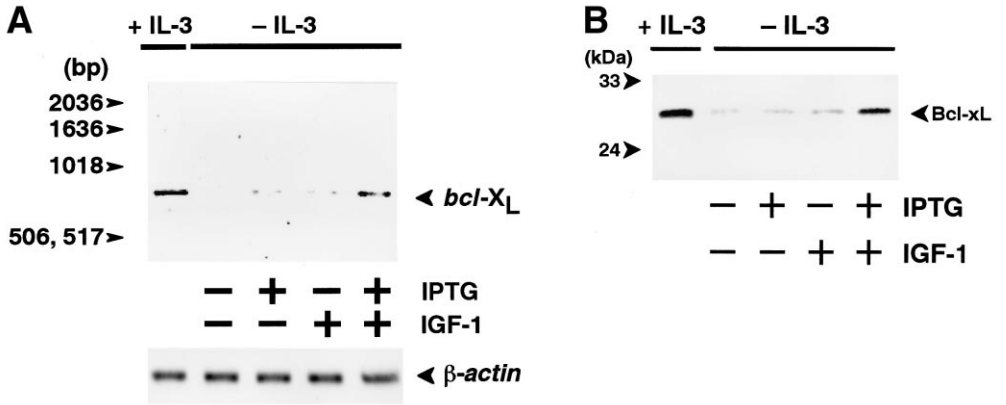

Fig. 3. Effect of R-Ras and IGF-1 on Bcl-xL expression. A: RT-PCR analysis of *bcl*-xL mRNA. After 3 h starvation, Lh9 cells were incubated for 3 h with IGF-1 (20 ng/ml) or IL-3 (25 ng/ml) in the presence or absence of IPTG (5 mM). RNA extraction and RT-PCR using *bcl*-x-specific primers were performed as described in Section 2. The same experiments with β-actin-specific primers confirmed that approximately equal amounts of RNA were used as a template in all samples. B: Western blotting analysis of Bcl-xL protein. Lh9 cells were cultured for 24 h in the serum-free medium either in the presence or absence of IGF-1 (20 ng/ml) and/or IPTG (5 mM). Cell lysates were subjected to Western blotting analysis with anti-Bcl-x antibody. Size markers are indicated on the left. An arrowhead on the right indicates the band due to *bcl*-xL mRNA (A) or Bcl-xL protein (B). The results shown represent at least three independent experiments.

PCR with *bcl*-x-specific primers. Although the *bcl*-x gene is known to have two splicing variants, *bcl*-xL and *bcl*-xS, they were easily distinguished from each other, since the size of the amplified DNA derived from *bcl*-xL mRNA (~700 base) is larger than that from *bcl*-xS (~500 base). The amount of *bcl*-xL mRNA apparently increased in IPTG-treated (i.e. R-Ras^{Q87L}-expressing) Lh9 cells stimulated with IGF-1 (Fig. 3A). Either R-Ras^{Q87L} or IGF-1 alone induced only a small increase of *bcl*-xL mRNA. We could not detect the band of *bcl*-xS under these conditions. We next examined whether the same was true for the expression of Bcl-xL protein. Western blotting analysis showed that Bcl-xL protein was highly expressed in Lh9 cells growing in the presence of IL-3 and serum, while the amount of Bcl-xL protein was remarkably reduced by 1-day starvation of IL-3 and serum (Fig. 3B). This decrease of Bcl-xL protein in the starved Lh9 cells was significantly suppressed by a combination of R-Ras^{Q87L} and IGF-1, whereas each alone could inhibit the decrease only slightly. These results suggest that R-Ras and IGF-1 synergistically increase the amount of Bcl-xL in both mRNA and protein levels.

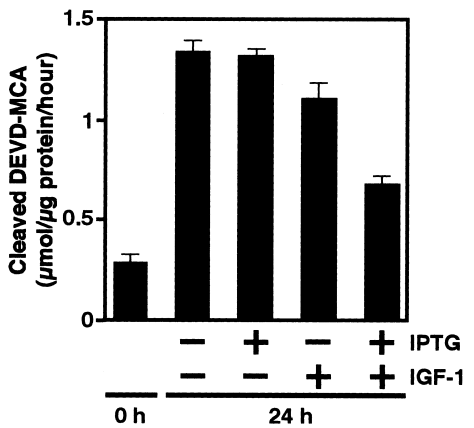

Fig. 4. Effect of R-Ras and IGF-1 on caspase-3 activation. Lh9 cells were cultured for 24 h in the serum-free medium with or without IGF-1 (20 ng/ml) and/or IPTG (5 mM). Caspase-3 activity in cell lysate was assessed using fluorogenic peptide (DEVD-MCA) as described in Section 2. Results are given as the mean ± S.E. of three separate studies.

3.3. Inhibition of caspase-3 activation by R-Ras and IGF-1

A recent report demonstrated that apoptosis of BaF3 cells on IL-3 deprivation exhibits a strong dependency on caspase-3 activation [25]. Therefore, we examined the effect of R-Ras and IGF-1 on caspase-3 activity using fluorescent peptide substrate (DEVD-MCA), which is relatively specific for this protease. The caspase-3 activity was maintained at low level in growing Lh9 cells (Fig. 4). However, after depletion of IL-3 and serum, caspase-3 activity gradually increased, and reached about 5-fold activation 24 h later. On this activation, IGF-1 displayed a slight inhibitory effect, while R-Ras did not show any effect. In the presence of IGF-1, however, R-Ras significantly suppressed caspase-3 activation. These data suggest that R-Ras and IGF-1 inhibit activation of caspase-3 in a cooperative manner.

4. Discussion

Our previous data suggest that both the PI3K-Akt pathway and the Raf-ERK pathway are required for BaF3 survival induced by R-Ras and IGF-1 [10]. However, we could not detect any activation of ERK by R-Ras in BaF3 cells. Though R-Ras has been reported to bind with Raf and activate ERK [2,3,7], it has recently been shown that the binding affinity of R-Ras to Raf is much lower than that of H-Ras [26], and that R-Ras induces only a marginal increase of ERK activity in Cos and HEK293 cells [4,10]. Therefore, it is likely that R-Ras had little effect on the activation of ERK in BaF3 cells. In contrast, Akt was strongly activated by R-Ras, which corresponds well with the recent report demonstrating Akt activation by R-Ras in Cos cells [4]. On the other hand, IGF-1 activated both Akt and ERK in BaF3 cells, although the level of activation was not so high in both cases. From these results, it is suggested that, when R-Ras and IGF-1 inhibit

apoptosis of BaF3 cells, the PI3K-Akt pathway is mainly activated by R-Ras, while IGF-1 is responsible for the activation of the Raf-ERK pathway. It has been reported that H-Ras suppresses the apoptosis of BaF3 cells even in the absence of serum [27,28], indicating that H-Ras, unlike R-Ras, does not require IGF-1. Considering that H-Ras activates the Raf-ERK pathway as well as the PI3K-Akt pathway, it is likely that one major role of IGF-1 in the R-Ras-induced suppression of cell death is to supplement R-Ras-exerted signals with the activation of the Raf-ERK pathway.

In the previous study, we demonstrated that IGF-1 supports R-Ras-induced BaF3 survival at a concentration of 20 ng/ml [10]. However, IGF-1 at this concentration could not induce detectable ERK activation (Fig. 1). Although these results raise the possibility that ERK activation by IGF-1 may not be important for cell survival induced by R-Ras and IGF-1, our previous finding that PD98059, a MEK inhibitor, partially suppresses survival of BaF3 cells by R-Ras and IGF-1 [10] strongly indicates that ERK activity somewhat contributes to BaF3 survival. We assume that this discrepancy is due to the difference in the conditions of the two experiments. In the case of ERK activation, cells were stimulated by IGF-1 at 25°C for only 10 min, whereas cells were incubated with IGF-1 at 37°C for a much longer period such as one day in the experiments of cell survival. Thus, a prolonged treatment of cells with 20 ng/ml IGF-1 at 37°C may induce ERK activation. In fact, our preliminary data indicate that, when stimulated with 20 ng/ml IGF-1 at 37°C for 2.5 h, Lh9 cells show higher ERK activity than that of unstimulated cells.

IGF-1 has been reported to induce Bcl-xL expression [23,29,30] and we also observed a slight induction of Bcl-xL by this growth factor (Fig. 3). The promoter region of the *bcl*-xL gene contains one AP-1-binding site [31], which is under the control of the Raf-ERK pathway. Moreover, it was reported that induction of Bcl-xL by IL-3 is dependent on ERK activation in BaF3 cells [23]. Thus, IGF-1 seems to stimulate Bcl-xL expression through ERK activation. As for R-Ras, to our knowledge there has been no report about its effect on Bcl-xL expression. In the present study, we observed a slight induction of Bcl-xL by R-Ras alone and the synergistic increase of Bcl-xL expression by R-Ras and IGF-1, indicating that R-Ras, as well as IGF-1, can induce the expression of Bcl-xL. Among downstream molecules of R-Ras, Akt has been shown to induce Bcl-2 expression in hematopoietic cells [32]. Since *bcl*-xL has a similar promoter organization to *bcl*-2 [31], it is possible that Akt is able to induce Bcl-xL expression. Taken together, it appears that R-Ras and IGF-1 synergistically increase Bcl-xL expression through activation of the PI3K-Akt pathway and the Raf-ERK pathway.

Bcl-xL has been shown to inhibit release of cytochrome *c*, an activator of caspase-3, from mitochondria [33]. Bcl-xL was also reported to associate with Apaf-1 to inhibit Apaf-1-dependent activation of caspase-9, another activator of caspase-3 [34]. These observations suggest that increase of Bcl-xL can result in the prevention of caspase-3 activation. In addition, although Bad is reported to down-regulate Bcl-xL through formation of heterodimer, activated Akt can induce disruption of this complex by phosphorylating Bad, which leads to production of free Bcl-xL [16,17]. Thus, inhibition of caspase-3 activation by R-Ras and IGF-1 seems to be a consequence of the increase of Bcl-xL expression and the release of Bcl-xL from the Bad·Bcl-xL complex.

A recent work by Thomas et al. suggests that overexpression of Bcl-xL is sufficient to inhibit the apoptosis of BaF3 cells [24]. Therefore, based on our present observations, the following model can be proposed for the mechanism of action of R-Ras and IGF-1 on survival of BaF3 cells. R-Ras and IGF-1 activate two distinct anti-apoptotic pathways: the PI3K-Akt pathway is activated by R-Ras and the Raf-ERK pathway by IGF-1. These pathways, combined, induce the up-regulation of Bcl-xL. Bcl-xL then inhibits caspase-3 activation, which leads to the suppression of apoptosis. Hematopoietic cells are usually exposed to a variety of cytokines, and their proliferation, differentiation and apoptosis are regulated by a combination of these cytokines. For example, a combination of IGF-1 and IL-7 stimulates proliferation of pro-B cells [35], while IGF-1 and erythropoietin induce erythroid maturation [36]. Thus, our results using BaF3 cells raise the possibility that a combination of IGF-1 and a cytokine that activates R-Ras leads to cell survival in certain types of hematopoietic cells. So far, it is unknown which cytokine(s) stimulates activation of R-Ras. RasGRF has been shown to be a possible exchange factor for R-Ras [37] and to be a downstream molecule of G-protein-coupled receptors [38]. Thus, R-Ras may be activated by some ligands for G-protein-coupled receptors. The present data also raise the possibility that R-Ras, when activated, can function as an apoptosis suppressor through activation of Akt in other cell types than hematopoietic cells, since Akt has been shown to be involved in survival of various kinds of cells [39]. We are currently investigating this possibility.

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