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Trophic support promotes survival of *bcl-x*-deficient telencephalic cells *in vitro*

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Abstract

Survival of immature neurons is regulated by Bcl-x_L, as targeted disruption of *bcl-x* significantly increases cell death *in vivo* and *in vitro*. Death of cultured *bcl-x*-deficient and wild-type telencephalic cells can be prevented by fetal calf serum or chemically-defined medium (ITS), suggesting trophic factors in these media potentiate survival through a pathway independent of Bcl-x_L. Addition of trophic factors to basal medium revealed that insulin and insulin-like growth factors (IGFs), but not other trophic factors, reduced apoptosis of wild-type and *bcl-x*-deficient telencephalic cells. Antibodies raised against IGF-I receptors and wortmannin both attenuated the effects of IGF-I, indicating survival was mediated by IGF-I receptors and phosphatidylinositol 3′-kinase signaling, whereas effects of ITS were only partially reduced by these agents. The survival promoting effects of ITS were reduced in cells lacking both *bcl-x* and *bcl-2*, indicating Bcl-2 plays a supportive role to Bcl-x_L in maintaining telencephalic cell survival. Furthermore, the ratio of expression of the pro-apoptotic *bax* gene to the anti-apoptotic *bcl-2* gene was reduced in *bcl-x*-deficient cultures grown in ITS, suggesting that the interaction between these *bcl-2* family members may, in part, regulate a Bcl-x_L independent survival pathway. Finally, the pro-apoptotic *bad* gene does not appear to play a role in these interactions as targeted disruption of *bad* did not alter apoptosis in telencephalic cultures.

Keywords: Bcl-x; Bcl-2; Bax; insulin; insulin-like growth factors; apoptosis; telencephalon

Abbreviations: BDNF, brain-derived neurotrophic factor; CNS, central nervous system; CNTF, ciliary neurotrophic factor; DMEM, Dulbecco's Modified Eagle's Medium; EGF, epidermal growth factor; FCS, fetal calf serum; GDNF, glial-derived neurotrophic factor; IGF, insulin-like growth factor; α-IR_3, anti-IGF-I receptor antibody; ITS, insulin-, transferrin-, and selenium-containing medium; MAP, microtubule associated protein; NGF, nerve growth factor; NT, neurotrophin; PCD, programmed cell death; PI3K, phosphatidylinositol 3′-kinase; S.E.M., standard error of the mean

Introduction

Significant numbers of cells are eliminated by programmed cell death (PCD) during nervous system development, with over 50% of neurons dying in some regions (Oppenheim, 1991). This death typically occurs by the process of apoptosis (Wyllie *et al*, 1980; Clarke, 1990). Classic studies of the nervous system have examined a period of PCD when neurons compete for limited amounts of trophic factors released by target tissues (Purves, 1986; Cowan *et al*, 1984; Levi-Montalcini, 1966; Oppenheim, 1991). However, a number of studies have demonstrated that widespread apoptosis occurs prior to target innervation in populations of immature neurons and neural precursor cells (Thomaidou *et al*, 1997; Galli-Resta and Ensini, 1996; Blaschke *et al*, 1996; Homma *et al*, 1994; Acklin and van der Kooy, 1993; Lance-Jones, 1982; Maruyama and D'Agostino, 1967). Although these cells are not yet dependent upon target-derived trophic factors, the possibility that survival of immature neurons and/or progenitor cells may be enhanced by non-target-derived trophic factors needs to be examined.

One gene that plays an important role in regulating survival of immature neurons prior to target innervation is *bcl-x*. Bcl-x is a member of a family of proteins which share sequence homology with Bcl-2 (Merry and Korsmeyer, 1997; Craig, 1995; Reed, 1994). Although *bcl-x* can be alternatively spliced into *bcl-*x_L or *bcl-*x_S, only *bcl-*x_L is expressed in the mouse CNS (Krajewski *et al*, 1994; Boise *et al*, 1993; Gonzalez-Garcia *et al*, 1994). Bcl-x_L prevents apoptosis of many cell types, including immune cells and post-mitotic neurons (Gonzalez-Garcia *et al*, 1995; Boise *et al*, 1993; Frankowski *et al*, 1995). Targeted disruption of *bcl-x* significantly increases apoptosis of immature neurons throughout the central nervous system (CNS), indicating that Bcl-x_L is necessary for the survival of these cells (Motoyama *et al*, 1995).

Primary cultures of E12 telencephalic cells have become a useful model in defining molecules important for early neuronal survival. Similar to neuroepithelial cells *in vivo*, wild-type E12 telencephalic cells cultured in basal medium can differentiate into neurons and/or die by apoptosis (Shindler *et al*, 1997). Furthermore, apoptosis in cultures of *bcl-x*-deficient (*bcl-*$x^{-/-}$) neurons significantly increases compared to wild-type cultures (Shindler *et al*, 1997; Roth *et al*, 1996). Interestingly, apoptosis of wild-type and *bcl-*$x^{-/-}$ telencephalic cells is almost completely eliminated in cultures grown in basal medium supplemented with fetal calf serum (FCS) or in chemically-defined medium

(ITS) containing a variety of compounds including insulin, transferrin, and selenium (Roth *et al*, 1996). These data suggest that factors present in these media promote survival of immature neurons.

In addition to Bcl-x_L, other Bcl-2 gene family members are important in controlling cell survival. Members such as Bcl-2, Bcl-w, and Mcl-1 inhibit apoptosis and promote cell survival, whereas Bax, Bad, and Bak promote apoptosis (reviewed in Merry and Korsmeyer, 1997). Some Bcl-2 family members can form heterodimers (Sato *et al*, 1994; Chittenden *et al*, 1995; Yin *et al*, 1994; Sedlak *et al*, 1995), and the balance between anti-apoptotic and pro-apoptotic proteins is important in determining if a given signal results in apoptosis (Shindler *et al*, 1997; Krajewski *et al*, 1995; Oltvai and Korsmeyer, 1994; Oltvai *et al*, 1993; Sedlak *et al*, 1995). Therefore, ITS and FCS may promote cell survival *in vitro* by regulating expression of the Bcl-2 gene family. For example, ITS and FCS may rescue *bcl-x*$^{-/-}$ telencephalic cells by promoting up-regulation of anti-apoptotic genes such as *bcl-2* or down-regulation of pro-apoptotic genes such as *bax*.

In the present study, survival of E12 telencephalic cells treated with neurotrophic factors was examined. Potential signaling of these factors through activation of IGF-I receptors and phosphatidylinositol 3′-kinase (PI3K) was examined by treating cultures with antibodies raised against IGF-I receptors (α-IR_3) and wortmannin, a PI3K inhibitor. Cultures of *bcl-2*$^{-/-}$/*bcl-x*$^{-/-}$ telencephalic cells were used to determine if ITS can maintain cell survival in the absence of *bcl-2*, and expression of *bcl-2* and *bax* mRNA were compared between cultures grown in basal medium or ITS. Furthermore, the role of *bad* was assessed in cultures of *bad*$^{-/-}$ cells. Results indicate that IGF-I, IGF-II, and insulin reduce apoptosis of telencephalic cells, and that ITS medium may, in part, promote survival by altering the balance between *bcl-2* and *bax*.

Results

ITS and FCS prevent apoptosis of wild-type and *bcl-x*-deficient telencephalic cells

Similar to results reported previously (Shindler *et al*, 1997), telencephalic cultures from E12 wild-type embryos grown for 48 h in basal medium (DMEM) contained differentiating neurons as determined by cellular morphology and expression of MAP2, a neuronal marker (data not shown). Cultures also contained a baseline level of cell death as $27.4 \pm 1.5\%$ of cells (from 24 embryos) were apoptotic (Figure 1A) as determined by condensed and fragmented chromatin staining with bisbenzimide. Cultures from *bcl-x*$^{-/-}$ mice (21 embryos) contained significantly more apoptotic cells ($76.5 \pm 1.7\%$ cells) (Figure 1A). Apoptosis of both wild-type and *bcl-x*$^{-/-}$ cells was significantly reduced in cultures grown in ITS or 1.0% FCS for 48 h (Figure 1A).

IGF receptor ligands promote telencephalic cell survival independent of Bcl-x_L

Since ITS and FCS have a dramatic survival promoting effect on cultured *bcl-x*$^{-/-}$ telencephalic cells, the ability of a variety of known neurotrophic factors to activate a Bcl-x_L independent anti-apoptotic pathway was examined. ITS contains a high

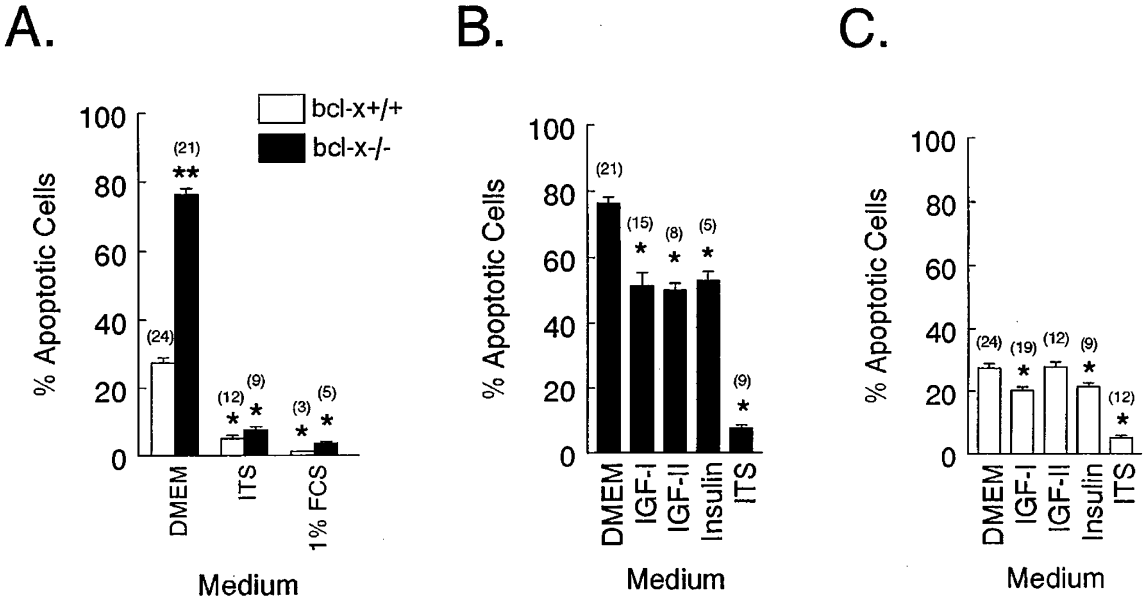

Figure 1 Enriched media, insulin, and insulin-like growth factors promote survival of wild-type (*bcl-x*$^{+/+}$) and *bcl-x*-deficient (*bcl-x*$^{-/-}$) E12 telencephalic cells in culture. (**A**) Chemically-defined medium containing insulin, transferrin, and selenium (ITS) and DMEM containing fetal calf serum (FCS) reduce the number of apoptotic cells as compared to cultures grown in DMEM. Data are expressed as mean percentage of cells with bisbenzimide-labeled condensed chromatin/total number of cells $\pm$S.E.M. Statistical significance ($P \leqslant 0.05$) is represented by * for comparisons to corresponding cultures in DMEM and ** for comparisons of cultures grown in DMEM. B-C. Insulin ($5\,\mu g\,ml^{-1}$) and insulin-like growth factors (IGF) ($100\,ng\,ml^{-1}$) decrease apoptosis of E12 cells from *bcl-x*$^{-/-}$ (**B**) and *bcl-x*$^{+/+}$ (**C**) mice as compared to sister cultures in DMEM (* denotes $P \leqslant 0.05$). Numbers in parentheses indicate the number of embryos examined in each condition

concentration of insulin which may activate IGF receptors; therefore, the ability of IGF receptor ligands IGF-I, IGF-II, and insulin to promote survival of *bcl-x*$^{-/-}$ cells was tested. Each of these ligands added to basal medium produced a significant reduction of the number of dying *bcl-x*$^{-/-}$ telencephalic cells (Figure 1B). Although IGF receptor ligands increased survival, apoptosis still occurred in approximately 50% of *bcl-x*$^{-/-}$ cells grown in supplemented media, suggesting that additional factors may promote survival of E12 *bcl-x*$^{-/-}$ telencephalic cells. The effects of a number of neurotrophic factors were tested using concentrations of factors shown previously to have maximal effects on other neuronal cells (Ip *et al*, 1994; Lin *et al*, 1993; Lindholm *et al*, 1996; Knusel *et al*, 1990; Widmer and Hefti, 1994). Addition of 50 ng ml^{-1} nerve growth factor (NGF), 25 ng ml^{-1} brain-derived neurotrophic factor (BDNF), 25 ng ml^{-1} neurotrophin (NT)-3, 25 ng ml^{-1} NT-4, 25 ng ml^{-1} ciliary neurotrophic factor (CNTF), 25 ng ml^{-1} glial-derived neurotrophic factor (GDNF), or 25 ng ml^{-1} epidermal growth factor (EGF) did not increase *bcl-x*$^{-/-}$ telencephalic cell survival (data not shown).

The above results suggest that IGF receptor ligands can promote telencephalic cell survival through a Bcl-x_L independent process. To determine whether these factors promote survival in the presence of Bcl-x_L, factors were added to wild-type telencephalic cell cultures and results were compared with those presented for *bcl-x*$^{-/-}$ cells. Similar to *bcl-x*$^{-/-}$ cultures, insulin and IGF-I significantly increased wild-type cell survival (Figure 1C). IGF-II failed to increase wild-type cell survival. NGF, BDNF, NT-3, NT-4/5, CNTF, GDNF, and EGF also failed to affect telencephalic cell survival (data not shown).

IGF-I promotes telencephalic survival through the IGF-I receptor and PI3K signaling

IGF-I has been shown previously to promote survival in some neuronal systems by activating the IGF-I receptor and PI3K signaling pathway (Singleton *et al*, 1996a,b; Matthews and Feldman, 1996; D'Mello *et al*, 1997). To determine if these pathways are similarly involved in IGF-I mediated survival of E12 telencephalic cells, cultures grown in 100 ng ml^{-1} IGF-I were treated with either 2 μg ml^{-1} α-IR_3 or 100 μM wortmannin, concentrations which have been used previously to block IGF-I receptor activation and PI3K, respectively (Matthews and Feldman, 1996; D'Mello *et al*, 1997). Differences in apoptosis were not found among wild-type or *bcl-x*$^{-/-}$ E12 telencephalic cultures grown in DMEM, DMEM and α-IR_3 or DMEM and wortmannin (data not shown). Cultures of *bcl-x*$^{-/-}$ cells grown in the presence of both IGF-I and either α-IR_3 or wortmannin contained significantly more apoptosis than cultures grown in IGF-I alone ($P \leqslant 0.05$), whereas levels of apoptosis were not different from cultures grown in unsupplemented DMEM ($P \geqslant 0.05$; Figure 2A). Similarly, in wild-type cultures there were no differences in apoptosis between cultures grown in unsupplemented DMEM and cultures treated with both IGF-I and α-IR_3 or with IGF-I and wortmannin ($P \geqslant 0.05$ Figure 2A).

ITS contains high concentrations of insulin that may be able to activate IGF-I receptors. To determine if IGF-I receptor activation and PI3K signaling are also involved in ITS mediated telencephalic survival, wild-type and *bcl-x*$^{-/-}$ cultures grown in ITS were treated with α-IR_3 or wortmannin. *bcl-x*$^{-/-}$ cultures grown in ITS supplemented with α-IR_3 or wortmannin contained significantly more apoptotic cells than cultures grown in unsupplemented ITS ($P \leqslant 0.05$; Figure 2B). Wild-type ITS cultures treated with α-IR_3 or wortmannin also showed a tendency toward increased apoptosis as compared to ITS cultures, but this difference was not statistically significant ($P \geqslant 0.05$; Figure 2B). However, apoptosis was still significantly lower in both wild-type and *bcl-x*$^{-/-}$ cultures grown in ITS with α-IR3 or wortmannin as compared to cultures grown in DMEM ($P \leqslant 0.05$; Figure 2B).

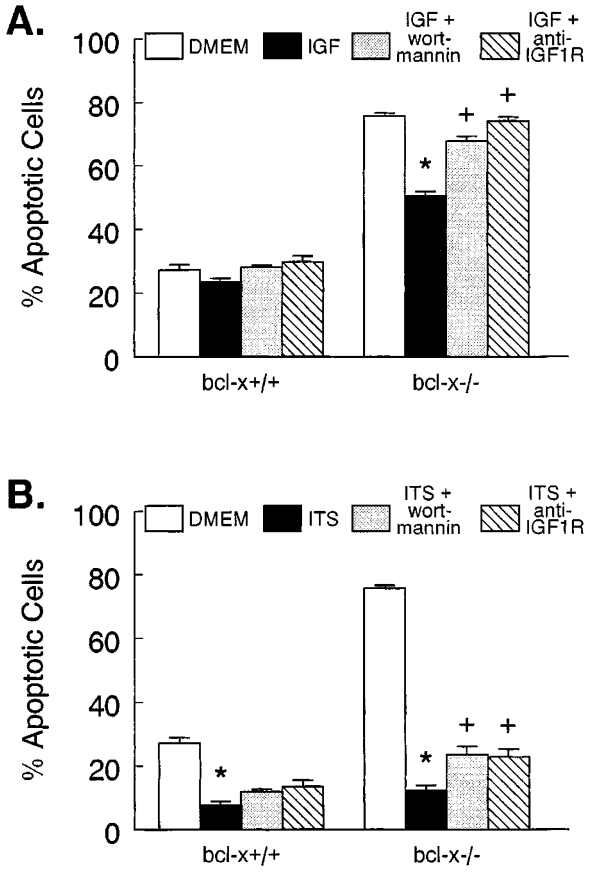

Figure 2 Wortmannin or antibodies raised against IGF-I receptors attenuate survival of *bcl-x*$^{-/-}$ E12 telencephalic cells in media containing IGF-I or ITS. Addition of antibodies raised against IGF-I receptors (α-IR_3) or wortmannin, an inhibitor of PI3K, attenuates survival of *bcl-x*$^{-/-}$ cells grown in DMEM supplemented with IGF-I (**A**) or grown in ITS (**B**). Data are expressed as mean percentage of cells with bisbenzimide-labeled condensed chromatin/total number of cells $\pm$SEM. Statistical significance ($P \leqslant 0.05$) is represented by * for comparisons to corresponding cultures in DMEM and + for comparisons of cultures grown in ITS or IGF-I

bcl-2 promotes survival of *bcl-x*$^{-/-}$ telencephalic cells in ITS

Insulin, IGF-I, and IGF-II reduced apoptosis of E12 *bcl-x*$^{-/-}$ telencephalic cells, although ITS and FCS appeared more

effective in promoting cell survival. In the absence of Bcl-x_L, other anti-apoptotic members of the Bcl-2 family might promote telencephalic cell survival. We hypothesized that ITS may reduce apoptosis of E12 telencephalic cells by regulating *bcl-2*, which could then function in place of Bcl-x_L to promote cell survival. Mice carrying a targeted disruption of *bcl-2* were bred to *bcl-x*-deficient mice to test this possibility. Double heterozygous (*bcl-x*$^{+/-}$/*bcl-2*$^{+/-}$) mice were raised and interbred to generate *bcl-x*$^{-/-}$/*bcl-2*$^{-/-}$ embryos. Wild-type (*bcl-x*$^{+/+}$/*bcl-2*$^{+/+}$) and *bcl-2*-deficient (*bcl-x*$^{+/+}$/*bcl-2*$^{-/-}$) cultures grown in DMEM did not contain differing amounts of apoptosis (Figure 3A). The increased apoptosis in *bcl-x*-deficient (*bcl-x*$^{-/-}$/*bcl-2*$^{+/+}$) cultures was also observed in double-deficient (*bcl-x*$^{-/-}$/*bcl-2*$^{-/-}$) cultures (Figure 3A). Furthermore, apoptosis in cultures generated from mice carrying a single copy of either *bcl-x* or *bcl-2* did not differ as compared to cultures from mice carrying two copies of the respective gene (data not shown).

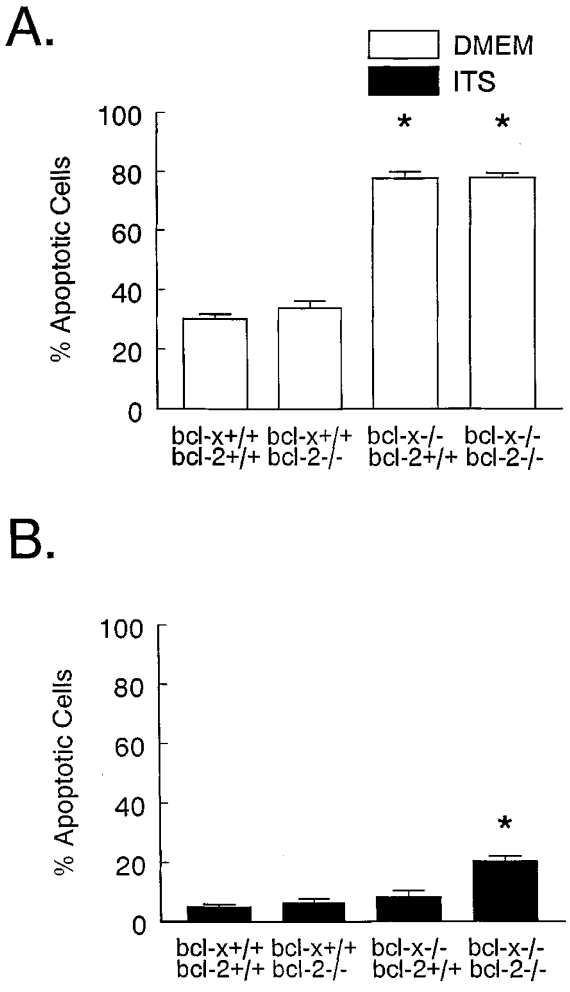

Figure 3 Disruption of *bcl-2* increases apoptosis in *bcl-x*-deficient cultures grown in ITS. (**A**) Disruption of *bcl-2* does not enhance apoptosis in *bcl-x*$^{+/+}$ or *bcl-x*$^{-/-}$ E12 telencephalic cultures grown in DMEM. Statistical significance ($P \leqslant 0.05$) is represented by * for comparisons to wild-type (*bcl-x*$^{+/+}$/*bcl-2*$^{+/+}$) cultures. (**B**) Disruption of *bcl-2* attenuates protective effects of ITS in *bcl-x*-deficient E12 telencephalic cultures. Statistical significance ($P \leqslant 0.05$) is represented by * for comparisons to wild-type (*bcl-x*$^{+/+}$/*bcl-2*$^{+/+}$) cultures. Data show the mean $\pm$SEM percentage of apoptotic cells in cultures generated from four *bcl-x*$^{+/+}$/*bcl-2*$^{+/+}$ embryos, five *bcl-x*$^{+/+}$/*bcl-2*$^{-/-}$ embryos, four *bcl-x*$^{-/-}$/*bcl-2*$^{+/+}$ embryos, and five *bcl-x*$^{-/-}$/*bcl-2*$^{-/-}$ embryos

Cultures grown in ITS contained significantly fewer apoptotic cells than those grown in DMEM (Figure 3). In ITS, the number of apoptotic cells was not different in cultures generated from *bcl-x*$^{+/+}$/*bcl-2*$^{+/+}$, *bcl-x*$^{+/+}$/*bcl-2*$^{-/-}$, or *bcl-x*$^{-/-}$/*bcl-2*$^{+/+}$ mice (Figure 3B). Although ITS significantly reduced apoptosis in *bcl-x*$^{-/-}$/*bcl-2*$^{-/-}$ cultures as compared to cultures grown in DMEM, survival promoting effects of ITS were attenuated by disruption of *bcl-2*. Cultures of *bcl-x*$^{-/-}$/*bcl-2*$^{-/-}$ cells grown in ITS contained significantly more apoptotic cells than either wild-type or *bcl-x*$^{-/-}$/*bcl-2*$^{+/+}$ cultures (Figure 3B), indicating *bcl-2* expression contributes to the Bcl-x_L independent survival pathway observed in *bcl-x*$^{-/-}$ telencephalic cells grown in ITS.

The ratio of *bcl-2* to *bax* is increased in E12 telencephalic cultures grown in ITS

The reduced ability of ITS to promote survival of *bcl-x*$^{-/-}$ cells lacking *bcl-2* suggests that ITS may exert its effects by regulating expression of *bcl-2* family members. Expression of the anti-apoptotic *bcl-2* gene and the pro-apoptotic *bax* gene was examined. *bax* has been shown previously to interact with *bcl-x* to promote survival of E12 telencephalic cultures (Shindler *et al*, 1997). Levels of *bcl-2* and *bax* mRNA were compared by semi-quantitative RT–PCR using the constitutively expressed *cyclophilin* gene to normalize the relative amount of mRNA in each culture. In both wild-type (Figure 4A) and *bcl-x*$^{-/-}$ (Figure 4B) cultures, *cyclophilin* expression was higher in cultures treated with ITS as compared to cultures grown in DMEM, reflecting increased cell survival in enriched medium. In wild-type cultures, ITS increased the ratio of *bcl-2* to *bax* mRNA to 1.35 ± 0.08 that of cells grown in basal medium (from six embryos, $P \leqslant 0.05$) (Figure 4A). An increased ratio of *bcl-2* to *bax* expression (1.44 ± 0.09) was also observed for *bcl-x*$^{-/-}$ cells grown in ITS *versus* DMEM (three embryos, $P \leqslant 0.05$) (Figure 4B), suggesting that ITS alters *bcl-2* and *bax* expression independent of the *bcl-x* gene. Interestingly, although an increased ratio of *bcl-2* to *bax* expression was observed in both wild-type and *bcl-x*$^{-/-}$ cells grown in ITS, wild-type cells contained decreased expression of both *bcl-2* and *bax* in ITS versus DMEM ($P \leqslant 0.05$) ($75.8 \pm 6.7\%$ and $56.1 \pm 3.5\%$ for *bcl-2* and *bax*, respectively). *bcl-x*$^{-/-}$ cells, however, showed no change in *bcl-2* expression ($P \geqslant 0.05$) but decreased *bax* expression ($P \leqslant 0.05$) ($104.8 \pm 12.3\%$ and $73.5 \pm 8.7\%$, respectively). Expression of *bcl-x* mRNA was not significantly altered by ITS in wild-type cultures ($P \geqslant 0.05$) ($87 \pm 16\%$), nor was it detected in *bcl-x*$^{-/-}$ cultures (Figure 4).

E12 telencephalic cell survival is not regulated by Bad

In addition to Bcl-x, Bcl-2, and Bax, other members of the Bcl-2 family may balance with these members to affect survival of

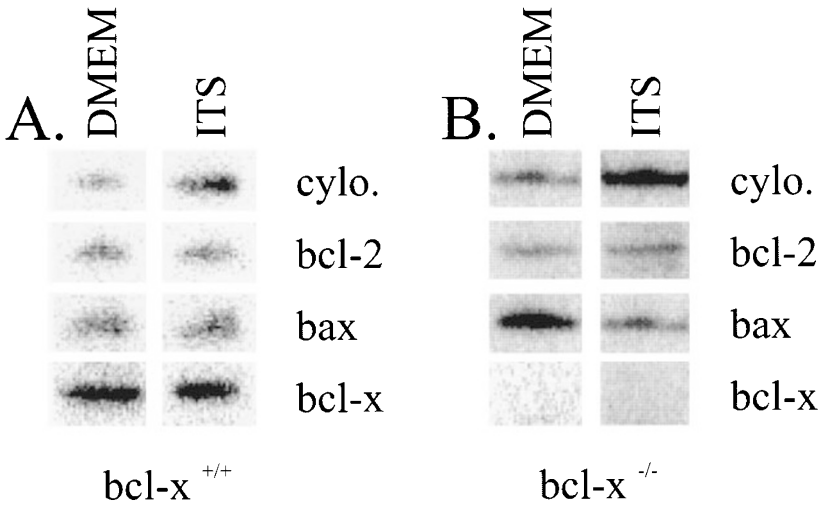

Figure 4 Analysis of *bcl-2* family mRNAs in E12 telencephalic cells by reverse transcriptase polymerase chain reaction. Wild-type (*bcl-x*$^{+/+}$) (**A**) and *bcl-x*-deficient (*bcl-x*$^{-/-}$) cells (**B**) were grown in DMEM or ITS, RNA was isolated, and cDNA was prepared. *Cyclophilin* and *bcl-2* family mRNAs were amplified by PCR; products were separated on polyacrylamide gels, and visualized by autoradiography and phosphorimaging using ImageQuant software from Molecular Dynamics. Results shown are from single experiments and are representative of results observed from six *bcl-x*$^{+/+}$ and three *bcl-x*$^{-/-}$ embryos

telencephalic cells. In some cell types, Bad heterodimerizes with Bcl-x_L and inhibits its anti-apoptotic effects (Yang *et al*, 1995). To determine if Bad regulates apoptosis of E12 telencephalic cells, cultures of wild-type, heterozygous, and *bad*-deficient cells were grown for 48 h in DMEM. No difference in apoptosis was found between *bad*$^{+/+}$ (eight embryos), *bad*$^{+/-}$ (13 embryos), or *bad*$^{-/-}$ (seven embryos) cultures ($P \geqslant 0.05$; Figure 5).

Discussion

The ability of ITS and FCS-supplemented DMEM to inhibit apoptosis of E12 telencephalic cells (Roth *et al*, 1996; and this study) indicates that factors present in these media promote survival of immature telencephalic cells. Since ITS contains a high concentration of insulin, it was hypothesized that insulin was important in mediating cell survival. This study demonstrates that insulin and insulin-like molecules IGF-I and IGF-II can inhibit apoptosis of E12 telencephalic cells. These results are supported by other studies illustrating that both IGF-I and IGF-II promote survival in many neuronal systems (Lindholm *et al*, 1996; D'Mello *et al*, 1993; Neff *et al*, 1993; Aizenman and de Vellis, 1987). The mechanism by which IGF-I mediates effects in other systems involves IGF-I receptor activation and PI3K pathways (Singleton *et al*, 1996a,b; Matthews and Feldman, 1996; D'Mello *et al*, 1997), and results presented here indicate a similar mechanism is used by immature telencephalic cells. IGF-I, IGF-II, and IGF-I receptors are expressed in the CNS by the early developmental period studied (Bondy *et al*, 1990; Rotwein *et al*, 1988), and activation of IGF-I receptors may therefore promote survival of immature telencephalic cells *in vivo*.

Although IGF receptor ligands reduced cell death, apoptosis was not reduced to the same extent as it was

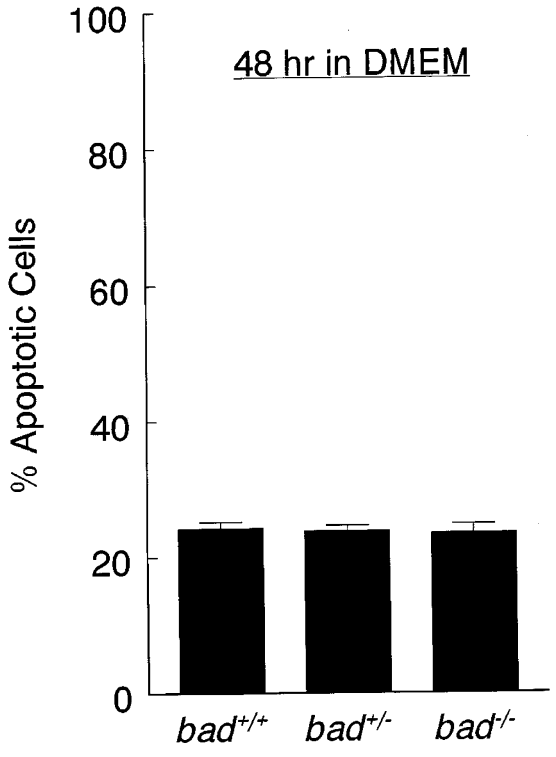

Figure 5 *bad* deficiency does not alter apoptosis of E12 telencephalic cells. Wild-type (*bad*$^{+/+}$), heterozygous (*bad*$^{+/-}$), and *bad*-deficient (*bad*$^{-/-}$) telencephalic cells were isolated on E12 and grown for 48 h in DMEM. Cells were fixed, stained with bisbenzimide, and the number of abnormal, apoptotic nuclei was counted as a percentage of the total number of nuclei present (% apoptotic cells). No differences in apoptosis were found between *bad*$^{+/+}$ (n=8), *bad*$^{+/-}$ (n=13), or *bad*$^{-/-}$ (n=7) cultures ($P \geqslant 0.05$)

by ITS or FCS, suggesting that additional factors may also promote telencephalic cell survival. A number of other factors, including NGF, BDNF, NT-3, NT-4/5, CNTF, GDNF, and EGF promote neuronal survival and differentiation in distinct cell populations. Specific effects on embryonic telencephalic cell populations have been reported. For example, BDNF promotes survival of primary cortical neurons *in vitro* (Ghosh *et al*, 1994), and NT-4/5 causes increased survival of developing striatal neurons (Garcia *et al*, 1992; Ardelt *et al*, 1994). The inability of these factors to promote survival in the present study may reflect the fact that few studies have used telencephalic cells isolated as early as E12. Furthermore, in many studies telencephalic cells were grown in FCS- or ITS-supplemented media prior to assessment of the effects of neurotrophic factors. Previously reported neurotrophic factor responsiveness may be a property of maturing neurons. Addition of FCS or ITS may promote survival of precursor cells *in vitro*, resulting in differentiation and eventual responsiveness to neurotrophic factors. The failure of neurotrophic factors to rescue E12 telencephalic cells in the current study may therefore indicate that neural precursors and/or progenitor cells have not yet developed the ability to respond to these factors.

The increased death of *bcl-x*$^{-/-}$ telencephalic cells as compared to wild-type cells demonstrates that these cells are kept alive by a pathway which is dependent on Bcl-x_L. The ability of IGF receptor ligands and enriched media to promote survival of *bcl-x*$^{-/-}$ cells, however, indicates these factors can stimulate at least one Bcl-x_L independent anti-apoptotic pathway. It has been shown that Bax interacts with Bcl-x_L to regulate telencephalic cell survival in the Bcl-x_L dependent pathway (Shindler *et al*, 1997), but the potential role of other Bcl-2 family members in promoting Bcl-x_L independent survival was not known. Death of immature telencephalic cells by both Bcl-x_L dependent and independent pathways is mediated by members of the caspase family of cysteine proteases (Roth and Shindler, 1998). Furthermore, this death occurs by an apoptotic process as evidenced not only by caspase activation, which occurs during apoptosis but not during necrosis (Armstrong *et al*, 1997), but also by nuclear condensation and terminal deoxytransferase-mediated deoxyuridine triphosphate nick end-labeling (Shindler *et al*, 1997; Roth *et al*, 1996), previously used markers of apoptosis (Deckwerth and Johnson, 1993; Gavrieli *et al*, 1992). Results here indicate that trophic factor signaling can indeed prevent this apoptotic process even in the absence of Bcl-x_L.

IGF-I signaling through the IGF-I receptor and PI3K may promote telencephalic cell survival through a pathway that is completely independent of the Bcl-2 family. Alternatively, this signaling pathway may directly regulate Bcl-2 gene family members. Furthermore, although ITS mediated survival was observed to be partially dependent on the IGF-I receptor and PI3K, ITS continued to exert a significant survival effect in the presence of α-IR_3 or wortmannin. Additional signaling pathways activated by ITS may circumvent the role of the Bcl-2 family or may directly regulate Bcl-2 family members. Although a signaling pathway that promotes survival by directly inhibiting the cell death machinery is most consistent with preventing death in response to an acute stimulus, the alternative hypothesis that ITS may also use a longer process of regulating *bcl-2* gene family member expression needs to be considered. In the paradigm used in these studies, apoptosis occurs approximately 30 h after initial plating of cells, whereas the ITS media is added at time zero. This may provide sufficient time for ITS to alter gene expression and thereby affect the susceptibility of a cell to an apoptotic signal.

Members of the Bcl-2 family form homo- and heterodimers (Sato *et al*, 1994; Chittenden *et al*, 1995; Yin *et al*, 1994; Sedlak *et al*, 1995), and the intracellular balance between pro- and anti-apoptotic members may ultimately determine the fate of a cell in response to a given signal (Krajewski *et al*, 1995; Oltvai and Korsmeyer, 1994; Oltvai *et al*, 1993; Sedlak *et al*, 1995). For example, the ratio of anti-apoptotic Bcl-x_L to pro-apoptotic Bax protein is important in regulating immature telencephalic neuron survival (Shindler *et al*, 1997). The ability of ITS, insulin, and IGFs to reduce apoptosis of *bcl-x*$^{-/-}$ telencephalic cells therefore suggests that these factors may act to restore a favorable intracellular balance of Bcl-2 family members. IGF-I does alter levels of some Bcl-2 family proteins in cultured neuroblastoma cells (Singleton *et al*, 1996a), indicating insulin-like molecules can alter the intracellular balance of Bcl-2 family members in some systems, although Bcl-2 family expression is not affected in other cell types rescued by IGF-I (Xu *et al*, 1997; Jung *et al*, 1996).

The ability of ITS to promote E12 telencephalic cell survival in the absence of *bcl-x* depends, in part, on *bcl-2* expression. Although ITS attenuated apoptosis in wild-type and *bcl-x*$^{-/-}$ cells, ITS could not prevent apoptosis of some *bcl-x*$^{-/-}$ cells lacking a functional *bcl-2* gene. This result suggests that ITS requires *bcl-2* to promote survival in at least a subpopulation of telencephalic cells, and is consistent with the possibility that ITS may restore a favorable balance between Bcl-2 family members. In previous studies, *bcl-2* deficiency showed only subtle nervous system abnormalities *in vivo* (Nakayama *et al*, 1993, 1994; Veis *et al*, 1993) despite the fact that Bcl-2 is present at high levels in proliferating neuroepithelial cells (Merry *et al*, 1994; Novack and Korsmeyer, 1994) and overexpression of *bcl-2* leads to increased brain weight and numbers of neurons in specific regions (Farlie *et al*, 1995; Martinou *et al*, 1994). Taken together, these results suggest Bcl-2 plays a secondary role to Bcl-x_L in the developing CNS, serving a redundant function to promote survival when *bcl-x* fails to be expressed.

Rescue of *bcl-x*$^{-/-}$/*bcl-2*$^{-/-}$ telencephalic cells by ITS may be reduced if ITS normally acts to up-regulate *bcl-2*. Alternatively, ITS may alter expression of other *bcl-2* gene family members and still require functional *bcl-2* to maintain a favorable balance of anti-apoptotic proteins. Data support the latter hypothesis because ITS decreased expression of the pro-apoptotic *bax* gene, without up-regulating *bcl-2*, resulting in a small, yet significant increase in the ratio of

bcl-2 to *bax*. In previous studies, the ratio of *bcl-2* to *bax* was shown to directly correlate with cell survival (Oltvai *et al*, 1993); therefore, an increased *bcl-2* to bax ratio may represent one mechanism by which ITS promotes telencephalic cell survival.

The relative proportion of *bax* decreased in both wild-type and *bcl-x*$^{-/-}$ cells, suggesting changes in *bax* resulted from treatment with ITS, rather than a cellular response to loss of *bcl-x*. The observation that *bax* is down-regulated by ITS assumes that levels of *cyclophilin*, a constitutively-active gene routinely used (Estus *et al*, 1994), remains constant within individual cells. Decreased levels of *cyclophilin* observed in DMEM *versus* ITS treated cultures may reflect the fact that many cells grown in DMEM were apoptotic. Unlike other systems used to study apoptosis, apoptotic cells in these low density telencephalic cultures are not removed by phagocytosis. As a result, the total number of cells does not change during the culture period such that DMEM and ITS cultures contain equivalent total cell numbers (Shindler *et al*, 1997). Because apoptotic cells have reduced mRNA synthesis (Johnson *et al*, 1996; Deckwerth and Johnson, 1993), the uncleared apoptotic cells in DMEM cultures most likely contribute reduced amounts of mRNA. Therefore, changes in expression observed in the current studies probably represent mRNA levels in viable cells, and strongly suggests that ITS can regulate *bcl-2* family expression.

Although ITS promoted a statistically significant increase in the ratio of *bcl-2* to *bax*, this change was still relatively small compared to the changes in this ratio seen to affect survival in other cell populations (Oltvai *et al*, 1993). The ability of ITS to regulate Bcl-2 family expression in immature telencephalic cells therefore may only partially, if at all, contribute to its survival promoting ability. Furthermore, the current studies focused mainly on the expression of *bcl-x*, *bcl-2*, and *bax* because previous studies of expression and function have indicated roles for these family members in the developing telencephalon. The possible role of other Bcl-2 family proteins could further alter the balance between pro- and anti-apoptotic members. One such member, Bad, has been shown to block the anti-apoptotic function of Bcl-x_L in some cell types (Yang *et al*, 1995). Bad does not, however, appear to be involved in regulating immature telencephalic cell survival because targeted disruption of *bad* did not cause any changes in apoptosis. Although a growing number of additional Bcl-2 family members have been identified, expression studies have not examined whether most of these genes are expressed in the E12 telencephalon. The current data presented indicate that ITS can at least affect expression of some *bcl-2* family genes, and the overall effects on the balance between all family members will have to be addressed as new members are found to be expressed and play a role in the developing telencephalon.

In summary, E12 telencephalic cells used in this study represent a population of neural precursor cells and immature neurons that have not yet innervated target tissues. Thus, extensive PCD occurring during this early development does not result from competition for target-derived trophic factors. Results of this study suggest that these early cells may depend on non-target-derived trophic factors. The IGFs represent one family that mediates these effects, and does so by promoting a Bcl-x_L independent survival pathway.

Materials and Methods

Trophic factors

Human recombinant IGF-I, IGF-II, and insulin, and purified EGF from mouse submaxillary glands were purchased from Sigma, St. Louis, MO, USA. Purified NGF 2.5S from mouse submaxillary glands and human recombinant GDNF were purchased from Alomone Labs, Jerusalem, Israel. Human recombinant CNTF, BDNF, NT-3, and NT-4 were generous gifts from Regeneron Pharmaceuticals, Tarrytown, NY, USA.

Generation and detection of *bcl-x*-, *bcl-2*- and *bad*-deficient mice

The use of homologous recombination in ES cells to generate *bcl-x*$^{-/-}$ (Motoyama *et al*, 1995) and *bcl-2*$^{-/-}$ (Nakayama *et al*, 1993, 1994) mice has been described previously. *bad*$^{-/-}$ mice were also generated by homologous recombination in ES cells. *bcl-x*$^{+/-}$ male and female mice were bred to generate wild-type, heterozygous, and *bcl-x*$^{-/-}$ embryos, and endogenous and disrupted genes were detected by PCR analysis of tail DNA extracts (Shindler *et al*, 1997). Similarly, *bad*$^{+/-}$ male and female mice were bred to generate wild-type, heterozygous, and *bad*$^{-/-}$ embryos, and endogenous and disrupted genes were detected by PCR. To generate *bcl-x*$^{-/-}$/*bcl-2*$^{-/-}$ mice, heterozygous *bcl-x*$^{+/-}$ and *bcl-2*$^{+/-}$ were first bred to yield *bcl-x*$^{+/-}$/*bcl-2*$^{+/-}$ double heterozygotes that were subsequently interbred. Endogenous and disrupted *bcl-2* were also detected by PCR of tail DNA extracts (Nakayama *et al*, 1993).

Primary telencephalic cultures

E12 telencephalic cells were dissociated as described previously (Shindler and Roth, 1996). In short, embryos were removed on gestational day 12, telencephalic vesicles were isolated, and cells were dissociated in 0.01% trypsin with 0.004% EDTA (Sigma) and 0.001% deoxyribonuclease I (Sigma), followed by mild trituration with a fire-polished Pasteur pipette. Cells were washed and resuspended in basal media [DMEM; a 1:1 mix of Dulbecco's Modified Eagle Medium and Ham's F12 Medium (Life Technologies, Grand Island, NY, USA) with 14 mM sodium bicarbonate and 15 mM HEPES, pH 7.4].

Twenty-thousand cells were plated in each well of a 48 well tissue culture plate. Wells were precoated with 0.1 mg ml^{-1} poly-L-lysine (Sigma) and 0.1 mg ml^{-1} laminin (Sigma) prior to plating. Cells were incubated for 48 h in 5% CO_2 at 37°C in DMEM alone, DMEM supplemented with neurotrophic factors or 1% FCS, or in ITS media [DMEM supplemented with 5 μg ml^{-1} insulin, 100 μg ml^{-1} transferrin, 30 nM selenium, 20 nM progesterone, 100 μM putrescine, 6 g/L glucose, and 2 mM glutamine (all chemicals from Sigma)]. For signaling studies, 2 μg ml^{-1} α-IR_3 (Oncogene Research Products, Cambridge, MA, USA) or 100 μM wortmannin (Sigma) was added to the culture media at the time of plating.

Bisbenzimide labeling and quantification of apoptosis

After 48 h *in vitro*, cells were fixed for 20 min at room temperature in PBS with 4% paraformaldehyde. Cell nuclei were stained with a 0.04 μg ml^{-1} solution of bisbenzimide (Hoechst dye 33258; Sigma) in PBS. Cell counts were done as previously described (Shindler *et al*, 1997). Briefly, numbers of total nuclei and abnormally condensed, fragmented (apoptotic) nuclei were counted in four random fields at 40 $\times$ magnification for each well, and duplicate wells were counted for each culture condition. The percentage of apoptotic cells was calculated by dividing the number of apoptotic nuclei by the total number of nuclei. Significance was established using Kruskal-Wallis analysis of variance followed by Dunn's test for all pairwise multiple comparisons.

Isolation of total RNA

Total RNA was isolated using Ultraspec^{TM} RNA isolation system (Biotecx Laboratories, Houston, TX, USA). After 48 h *in vitro*, plates were placed on ice and medium was gently aspirated and replaced with 300 μl Ultraspec^{TM} RNA solution. Cells were lysed by trituration through a pipette tip. Lysates were transferred to 1.5 ml Eppendorf tubes on ice and lysates from duplicate cell cultures were combined (such that 40 000 cells were used in each condition). After 5 min, 120 μl chloroform was added to each tube, samples were mixed vigorously and incubated on ice for 5 min. Samples were spun 15 min at 13 500 r.p.m. at 4°C and each aqueous phase was transferred to a new tube. RNA was precipitated with an equal volume of isopropanol for $\geqslant$1 h at $-$20°C. RNA was pelleted at 13 500 r.p.m. for 15 min, then washed twice with 70% EtOH. After the final wash, RNA was resuspended in 50 μl DEPC-treated water and stored at $-$80°C until used.

Semi-quantitative reverse-transcriptase polymerase chain reaction (RT–PCR)

cDNA was generated from 25 μl of each RNA sample using Ready-To-Go^{TM} You-Prime First-Strand beads (Pharmacia, Uppsala, Sweden) following manufacturer's instructions. After heating RNA for 10 min at 65°C, samples were chilled to 4°C. Samples were then transferred to a tube with a Ready-To-Go bead containing Moloney Murine Leukemia Virus Reverse Transcriptase. After addition of 0.2 μg random hexamer primers (Boehringer Mannheim, Indianapolis, IN, USA), cDNA was synthesized at 37°C for 60 min.

The *bcl-2*, *bax*, and *bcl-x* primer sequences were identical to those used previously (Greenlund *et al*, 1995) and generated 231, 129 and 337 bp products, respectively. The *cyclophilin* forward (5′-GGGTTCCTCCTTTCACAGA-3′) and reverse (5′-GCCATCCAGC-CATTCAGTC-3′) primers yielded a 226 bp fragment. Each PCR reaction used 0.8 μl of cDNA in a 20 μl solution containing 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 0.1 mM each of dATP, dGTP, and dTTP; 0.05 mM dCTP; 0.5 μCi [α-^{32}P]-dCTP (NEN Life Sciences, Boston, MA, USA); and 0.4 U Taq polymerase (Perkin-Elmer, Norwalk, CT, USA), final concentrations. Each reaction also contained 2 μM cyclophilin primers or 0.8 μM *bax*, *bcl-2*, or *bcl-x* primers. After a hot start at 94°C for 1 min, reactions were cycled at 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min. The number of PCR cycles was chosen so that product formation remained within a linear range (Freeman *et al*, 1994; Estus *et al*, 1994). *Cyclophilin* was amplified for 22 cycles, *bcl-2* for 27 cycles, *bax* for 25 cycles, and *bcl-x* for 27 cycles in a Perkin-Elmer GeneAmp PCR System 2400. PCR products were separated on an 8% polyacrylamide gel, dried, and visualized on a PhosphorImager using ImageQuant software for volume integration (Molecular Dynamics, Sunnyvale, CA, USA). Changes in the ratios of *bax*, *bcl-2*, or *bcl-x* expression divided by *cyclophilin* expression were examined by comparing ratios from DMEM cultures with products amplified from ITS cultures. Significance of changes in mRNA levels were determined using a Signed Ranks Test.

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