**Fas Drives Cell Cycle Progression in Glioma Cells via Extracellular Signal-regulated Kinase Activation**

Hisaaki Shinohora, Hideo Yagita, Yoji Ikawa, and Naoki Oyaizu

Department of Retroviral Regulation, Medical Research Division, Tokyo Medical and Dental University, Tokyo 113-8519 [H. Y.; I. N. O.], and Department of Immunology, Juntendo University School of Medicine, Tokyo 113-8421 [H. Y.], Japan

**ABSTRACT**

Recent studies have revealed that a variety of malignant tumors express Fas and/or its ligand FasL. However, tumors cells expressing Fas are not always susceptible to Fas-mediated cell death, and the biological significance of simultaneous expression of Fas and FasL in the same tumor is not known. In the present study, we addressed this question in three glioma cell lines, A-172, T98G, and YKG-1, which express both Fas and FasL endogenously and their Fas transfectants. We report here that: (a) in gliomas, [3H]TdR incorporation was enhanced by anti-Fas IgM monoclonal antibody CH-11 and conversely inhibited by anti-Fas monoclonal antibody NOK-2; (b) cross-linking of Fas with CH-11 drove both cell cycle progression and apoptosis as demonstrated by the induction of the S-G2 phase of DNA and RNA and fragmented nuclei; (c) phosphorylation of extracellular signal-regulated kinase (ERK), but not of c-Jun NH2-terminal kinase or p38, was induced by cross-linking of Fas; (d) a mitogen-activated protein kinase/ERK kinase 1 (MEK1) inhibitor PD98059 completely blocked CH-11-induced ERK phosphorylation as well as cell cycle progression without affecting induction of apoptosis; and (e) a broad-spectrum caspase inhibitor Z-Asp-CH2-DCB inhibited CH-11-induced ERK phosphorylation, cell cycle progression, and apoptosis. These results indicate that Fas-mediated caspase activation elicits two independent cellular responses: one is to induce apoptosis and another is to promote cell cycle progression; the latter is closely linked to the MEK-ERK pathway. Together, our data strongly suggest that FasL may play a role as an autocrine growth factor in gliomas.

**INTRODUCTION**

Fas (also termed CD95 and APO-1) is a cell surface receptor that induces apoptosis in sensitive cells upon oligomerization by the interaction with its ligand FasL. The primary function of the Fas-FasL system is thought to be the maintenance of peripheral tolerance and lymphoid homeostasis (1, 2). Fas is expressed in a variety of primary as well as transformed cells of lymphoid or nonlymphoid origin. FasL expression was previously thought to be restricted to the immune effector cells, but subsequent studies have revealed that some stromal cells in the immune privileged sites (3, 4) and several malignant cells (5–8) constitutively express FasL. The significance of FasL expression in tumors has been implicated that it may promote evasion of tumors by eliminating the host antitumor response mediated by Fas-positive effector lymphocytes (5, 6).

The Fas-mediated signal transduction pathways have been extensively studied over the last several years. The Fas-mediated signaling cascade ultimately results in intracellular proteolysis and death (reviewed in Refs. 1 and 9). An alternate signaling pathway downstream of Fas involves the MAPK family members. Three distinct but related kinase cascades have been identified: the ERKs, the JNKs/stress-responsive MAP kinases, and the p38 MAPK. ERK is primarily activated by mitogens and growth factors, whereas JNK and p38 are stimulated by various stresses. Some previous studies showed that Fas triggered activation of MAPK, including JNK, p38, and ERK (10–16). In Jurkat T cells, Fas cross-linking triggered JNK and p38 activation in a caspase-dependent manner (10–12, 16). During Fas-mediated apoptosis, caspases cleaved and activated PAK2 (15) and MAPK/ERK kinase 1 (12), two kinases that can activate the JNK pathway. Fas cross-linking was also shown to activate ERK in SHEP cells (14), presumably via activation of the Ras pathway. Moreover, the additional connection of the Fas receptor to the stress-activated kinase pathway has recently been elucidated that upon Fas ligation, Daxx, a novel Fas-binding protein, interacts with and activates a MAP kinase kinase kinase termed ASK1, leading to the activation of the JNK and p38 MAPK pathways (17). Collectively, Fas can activate MAPK pathways by divergent mechanisms, some of which are caspase-dependent or independent. However, the biological role of MAPK activation downstream of Fas is presently controversial and needs further clarification.

Ligation of Fas with FasL does not solely transmit a death signal but instead may induce cell proliferation. In naive T lymphocytes, for example, Fas provided a costimulatory signal for proliferation yet also induced apoptosis in repeatedly stimulated T lymphocytes (18). Fas ligation has also been shown to enhance the growth of some tumor cell lines (19). However, the signaling mechanisms for proliferative effects of Fas ligation have not been clarified yet. Human gliomas are the most common brain tumors that possess unique characters with respect to cytokine sensitivities. These tumors express tumor necrosis factor α and its receptors and furthermore, proliferate in response to this cytokine (20, 21). It has been reported and we now show that some glioma cells express both Fas (22) and FasL (8), but the pathophysiological role of this endogenously expressed Fas/FasL interaction is not known. Although expressing substantial levels of Fas, glioma cells are generally resistant to cell death induced by anti-Fas treatment (22). Here, we show that endogenous Fas/FasL interaction provides a growth, rather than cell death, signal in glioma cells. Further, using Fas-transfected gliomas, we show evidence that Fas definitely transmits a cell cycle progression signal that is closely linked to ERK activation. We propose an additional role of Fas-FasL in tumorigenesis, that is, some tumors may make use of this system not only for evading immune surveillance but also for its own growth.

**MATERIALS AND METHODS**

**Cells and Cell Culture Condition.** Human glioma cell lines (A-172, T98G, and YKG-1) were obtained from Health Science Research Resources Bank (Osaka, Japan) and cultured in DMEM or MEM supplemented with 10% FCS. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. 1 Received 10/4/99; accepted 1/19/00. 2 The abbreviations used are: MAPK, mitogen-activated protein kinase; mAb, monoclonal antibody; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH2-terminal kinase; zD, Z-Asp-CH2-DCB; Rh123, rhodamine 123; PI, propidium iodide; 7AAD, 7-amino-actinomycin D; PY, pyronin Y; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MAP, mitogen-activated protein; MEK, MAPK/ERK kinase.
Fas PROMOTES GLIOMA CELL GROWTH VIA ERK

Fig. 1. Expression and function of Fas and Fasl on glioma cells. A, cell surface expression of Fas (top) and Fasl (bottom) on three glioma cell lines (A-172, T98G, and YKG-1) was examined by immunofluorescent staining with FITC-UB-2 and PE-NOK-2, respectively. Thin lines represent isotype controls. B, cytotoxic activity of FasL on glioma cells. Cytotoxic activities of glioma cells were tested against WR19L/Fas target cells by using nucleic acid dyes, 7AAD, and PY, which enable quantification of cell killing. % viability = [(cpm without effector − cpm with effector)/cpm without effector] × 100.

Cell Cycle Analysis. Cells were seeded onto a 24-well culture dish and [3 H] TdR (Amersham Pharmacia Biotech, Buckinghamshire, England) was added at the given E/T ratios. After culture for 16 h, intact DNA was collected by precipitation with trichloroacetic acid and was washed through the membrane filter (Millipore Corporation, Bedford, MA). Specific uptake of [3 H] TdR was measured using a microplate reader. Cell viability after CH-11 treatment was calculated as follows: % viability = [(A with CH-11 − A without CH-11)/A without CH-11] × 100.

Whole cell lysates (0.5 to 5 μg) were prepared and analyzed by Western blotting as described previously (25). Briefly, the full-length human Fas cDNA (provided by D. Schaal, Universitätsklinikum Schleswig-Holstein, Schwerin, Germany) was cloned into a MuLV retroviral vector PLXSN that contains neomycin-resistant gene as a selection marker. This construct was then transfected into the amphotropic packaging cell line PA317 with a commercially available kit (SuperFect, Qiagen, Hilden, Germany). Supernatant from the PA317 was used to infect three glioma cell lines, A-172, T98G, and YKG-1, respectively, as described (24). In brief, cells were incubated with Rh123 at 5 μg/ml for 30 min at 37°C, washed with PBS, and resuspended in PBS containing PI at 2 μg/ml. Rh123 and PI staining were analyzed on a flow cytometer (FACS Calibur, Becton Dickinson, San Jose, CA) using CELLQuest software (Becton Dickinson). Trypan blue exclusion was also used for counting of live cells under microscopy.

Transfection. Preparation and transfection of retrovirus were performed as previously described (25). Briefly, the full-length human Fas cDNA (provided by Dr. S. Nagata, Osaka University, Japan) was digested by XhoI and inserted into a MuLV retroviral vector PLXSN that contains neomycin-resistant gene as a selection marker. This construct was then transfected into the amphotropic packaging cell line PA317 with a commercially available kit (SuperFect, Qiagen, Hilden, Germany). Supernatant from the PA317 was used to infect three glioma cell lines, A-172, T98G, and YKG-1, respectively, in the presence of G418 (500 μg/ml). Two weeks after infection, each G418-resistant colony was picked up and expanded as a clonal population. Vector controls from each cell line that contain PLXN backbone were prepared in the same way.

Immunofluorescent Staining and Flow Cytometry. For study of cell surface expression of Fas and Fasl, cells were stained with FITC-conjugated anti-Fas mAb (clone UB2; MBL) and phycoerythrin-conjugated anti-Fasl mAb (NOK-2; Ref. 23). FITC- or PE-conjugated isotype control IgGs were obtained from PharMingen. The stained cells were analyzed on a flow cytometer.

Cell-mediated Cytotoxic Assay. A human Fas-transfected murine lymphoma cell line (WR19L/Fas) was used as the target cells (23). Cytotoxic activities of glioma cells were estimated by measuring target cell DNA fragmentation using the JAM test as described (5). In brief, the effector glioma cells were seeded onto a 24-well culture dish and [3 H] TdR (Amersham Pharmacia Biotech, Buckinghamshire, England) was added at the given E/T ratios. After culture for 16 h, intact DNA was collected by precipitation with trichloroacetic acid and was washed through the membrane filter (Millipore Corporation, Bedford, MA). Specific killing was calculated as follows: [(cpm without effector − cpm with effector)/cpm without effector] × 100.

Cell Proliferation Assay. Cells were seeded in a 48 well dish at a concentration of 1 × 10^4 cells/well. After 16-h incubation, medium was removed, and the cells were cultured with fresh medium containing 1 μCi/ml of [3 H] TdR and 100 ng/ml CH-11 for 24 h as described (20).

Quantitation of Cellular DNA and RNA Contents. Cell cycle analysis was performed using nucleic acid dyes, 7AAD, and PY, which enable quantitation of the content of DNA and RNA separately as described (26). In brief, trypsin-detached cells were suspended in nucleic acid staining solution, then stained with 50 μl of 400 μM 7AAD and 50 μl of 100 μM PY for 30 min. The stained cells were analyzed on a flow cytometer using CellQuest software (Becton Dickinson).

Immunoblotting for MAPKs. Whole cell lysates (0.5 to 5 × 10^6 cells/lane) were resolved in 2.5% SDS buffer and were applied on 12% PAGE and then electroblotted onto polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). After blocking with 5% nonfat dry milk in TBST (20 mM Tris (pH 7.4), 150 mM NaCl, and 0.1% Tween 20) for 30 min, the membranes were probed with the following antibodies: anti-ERK1, anti-p38 or anti-JNK2 (Santa Cruz, CA), or anti-phosphoThr202/Tyr204 MAPK, anti-phosphoThr183/Tyr185-JNK, or anti-phosphoThr71-p38 (New England Biolabs, Schwalbach, Germany). The bound antibodies were visualized by using PhoStone-Western Blot Detection kit (New England Biolabs). The bound antibodies were visualized by using PhoStone-Western Blot Detection kit (New England Biolabs).

Statistical Analysis. Statistical significance was assessed by Student’s t test, and P < 0.05 was considered as significant. Composite treatments were analyzed by Microsoft Excel Software.
Expression and Function of Fas and FasL on Glioma Cells. We first examined the cell surface Fas and FasL on three glioma cell lines, A-172, T98G, and YKG-1 by immunofluorescent staining (Fig. 1A). All of the cell lines examined expressed substantial but varying levels of surface Fas and FasL; Fas expression was highest on T98G and relatively low on A-172 and YKG-1. FasL expression was highest on YKG-1, followed by A-172 and T98G. To examine whether the FasL expressed on these cell lines is functional, we exogenously added an agonistic anti-Fas mAb (CH-11) to trigger a Fas-mediated signal or a neutralizing anti-FasL mAb (NOK-2) to block endogenous Fas-FasL interaction and estimated proliferation by [3H]Tdr incorporation. [3H]Tdr incorporation of YKG-1 cells was substantially augmented by CH-11 and conversely inhibited by NOK-2 (Fig. 1C). These unexpected observation suggested that glioma cells may use endogenous Fas/FasL for their own growth in an autocrine manner. We examined whether this augmented [3H]Tdr incorporation after the CH-11 treatment results in actual increase in cell number; however, the addition of CH-11 did not result in either increase or decrease of net live cell counts in glioma cell lines (not shown). These results prompted us to speculate that Fas may mediate both growth-promoting and death-inducing signals in glioma cells, and these opposite effects offset each other and thus resulted in seemingly no change in live cell counts.

Effect of CH-11 on Fas-transfected Glioma Cells. To further address the role of Fas/FasL in regulating tumor cell growth, we established stable full-length Fas transfectants from each cell line, which expressed high levels of surface Fas (Fig. 2A). To assess the effect of CH-11 on the growth of Fas transfectants, we initially estimated the viability of the transfectants after 24-h culture with various doses of CH-11 by a modification of the MTT assay. As shown in Fig. 2B, viability of all of the transfectants examined were substantially reduced by CH-11 at >0.1 μg/ml. In contrast, vector controls did not show such a reduced viability, and rather a slight increase was noted in T/M1 and Y/M1. No difference was observed between the vector controls and the untransfected parental cell lines.
(not shown). These results indicate that as a result of Fas overexpression, the transfectants might gain increased sensitivity to cell death. To directly determine the Fas-induced cell death, change in mitochondrial membrane potential (Δψm) and plasma membrane integrity were simultaneously analyzed by flow cytometry after dual staining with Rh123 and PI. As shown in Fig. 2C, CH-11 did induce cell death in transfectants as detected by an increase in PI-positive dead cells and Rh123-dim-positive apoptotic cells with reduced Δψm (24). To examine whether this reduction of cell viability might result in a decrease of cell number, we determined net live cell counts by trypan blue exclusion. As shown in Fig. 3, net live cell numbers of transfectants A/FF222 and T/FF14 at 24–72 h were not significantly different regardless of the presence or absence of CH-11. Although the growth of Y/FF014 was significantly suppressed by CH-11, it was notable that live cell counts were still increasing from the initiation of culture.

In a marked contrast, the addition of CH-11 to Jurkat T cells, a representative Fas-sensitive cell line, resulted in a progressive loss of live cells, and virtually no live cells could be detected by 72 h. These results indicated that Fas-expressing glioma cells still grow in the presence of CH-11 while a substantial part of cells are constantly depleted by Fas-mediated apoptosis. We interpreted these paradoxical results to suggest that Fas might promote proliferation of glioma cells, which surpassed the induced rate of apoptosis and thus resulted in an overall increase of cell number.

Fas PROMOTES GLIOMA CELL GROWTH VIA ERK

CH-11-promoted RNA Synthesis and Cell Cycle Progression of Glioma Cells. To examine the above possibility, we conducted cell cycle analysis using the 7AAD/PY method, which permits simultaneous quantitation of DNA and RNA contents in a cell. In preliminary experiments, we noticed that serum present in the culture largely mask the effects of CH-11 on cell cycle progression. To exclude this serum-derived effect, we precultured the cells in serum-free medium and then examined the effect of CH-11. As shown in Fig. 4A, serum starvation for 24 h could successfully arrest almost all A/FF222 cells at the G0 phase (as defined by diploid DNA with basal RNA expression). The serum deprivation per se did not significantly affect cellular viability of glioma cells, except that Y/FF014 showed slight decrease in viability, for the culture period up to 96 h (Table 1 and not shown).

In this condition, the CH-11 treatment for 24 h did result in cell cycle progression as manifested by the emergence of cells at the S-G2-M phase of the cell cycle, with high levels of induced RNA expression. The serum deprivation per se did not significantly affect cellular viability of glioma cells, except that Y/FF014 showed slight decrease in viability, for the culture period up to 96 h (Table 1 and not shown).

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and simultaneous emergence of cells with sub-G₀/G₁-fragmented DNA content indicative of apoptotic cells (Fig. 4B). As summarized in Table 1, all three parameters, the emergence of apoptotic cells, the S-phase entry, and the induction of RNA expression, were significantly driven by CH-11 in all of the transfectants derived from three glioma cell lines. Collectively, these results clearly indicated that Fas not only mediates apoptosis but also drives cell cycle progression.

**Fas-mediated Signaling Primarily Activated ERK.** In addition to caspases, MAPK cascades were shown to be activated downstream to Fas. To examine whether and which MAPKs are activated by Fas in glioma cells, we analyzed the phosphorylation of ERK, JNK, and p38 kinase in CH-11-stimulated glioma cells by immunoblotting. As shown in Fig. 5, after treatment with CH-11 for 15 min, a phosphorylated form of ERK (Lane 2, top), but not of JNK or p38, was detected. As a positive control, phosphorylated forms of ERK, JNK, and p38 were detected in the UV-irradiated cells (Lanes 6). We could detect this phosphorylated form of ERK in the absence of that of JNK and p38 for a period up to 60 min after CH-11 stimulation (Lane 3). The phosphorylation of ERK by CH-11 was completely inhibited by a MEK1 inhibitor PD98059 at a concentration of 10 μM (Lane 4). Furthermore, a caspase inhibitor zD could also block the phosphorylation of ERK (Lane 5).

**Effects of MEK Inhibitor and Caspase Inhibitor on Cell Cycle Regulation and Apoptosis.** To determine which signaling events are linked to the two dichotomous effects, we examined the effects of these inhibitors on cell cycle progression and apoptosis induction in CH-11-stimulated A/FF222 glioma cells. As shown in Fig. 6, the addition of PD98059 completely blocked the CH-11-driven S-phase entry and RNA induction but did not affect the apoptosis. The caspase inhibitor zD blocked the CH-11-induced apoptosis, as expected, and also inhibited the CH-11-driven S-phase entry and RNA induction. The addition of control IgM or each inhibitor alone did not affect the cell cycle progression or the apoptosis. These results suggested that Fas-mediated activation of the MEK-ERK pathway is closely linked to the Fas-driven cell cycle progression but not to the induction of apoptosis. Combined with the immunoblotting data that zD also blocked the CH-11-induced ERK activation, ERK is placed downstream of caspase(s) that transmit a cell growth signal by activating the MEK-ERK pathway in addition to mediating cell death.

**DISCUSSION**

The MEK-ERK pathway has been shown to be essential for cell proliferation by promoting G₁ cell cycle progression (27, 28). Ectopic expression of a constitutively active form of MEK1 could transform mammalian cells to a cancerous phenotype (29, 30). Sustained activation of the MEK-ERK pathway was also suggested to contribute to the neoplastic phenotype of malignant gliomas (31). In accordance with this, we observed a substantial level of steady state ERK phos-
phosphorylation in growing glioma cells cultured in the serum-containing medium, hence masking the effects of Fas. In the presence of serum, the addition of CH-11 further drives cell cycle progression and ERK phosphorylation, but these effects were inconspicuous probably due to powerful growth-promoting effects of serum-derived factor(s). However, when cultured under the serum-free condition, ERK phosphorylation became no longer detectable (Fig. 4, Lane 1), which was associated with the cell cycle arrest in the G0 phase. Here, we clearly demonstrated that stimulation of Fas drives cell cycle progression and ERK phosphorylation in serum-starved glioma cells. The Fas-driven cell cycle progression and ERK phosphorylation were completely blocked by an MEK1 inhibitor PD98059. These results indicate that glioma cells use the MEK-ERK pathway downstream of Fas for proliferation. We believe our present observation may represent a common feature of gliomas because similar results were obtained with three different glioma cell lines and their transfectants.

Because activation of JNK and p38 has been reported during the Fas-induced apoptosis in Jurkat T cells (10, 11, 16), our present observation of ERK phosphorylation in the absence of JNK or p38 phosphorylation in anti-Fas-stimulated glioma cells was somewhat unexpected. Ras activation, which potentially leads to ERK activation, has been implicated in the Fas-induced apoptosis in Jurkat cells (32), but other investigators have observed that Fas cross-linking does not result in ERK activation in this cell line (10, 33). In contrast, it was reported that both ERK and JNK were activated after Fas cross-linking in SHEP cells, a human neuroblastoma cell line (14). Utilization of MAPK subfamilies downstream of Fas thus appears to be divergent, and these differences are at least partly depending on the cell types used. Although we do not formally rule out the possibility that Fas mediates JNK or p38 activation in gliomas, it is worth noting that a p38 inhibitor SB 203580 had no effect on cell cycle progression or apoptosis in the anti-Fas-stimulated glioma cells at concentrations up to 10 μM. In any case, the upstream events that connect Fas to ERK activation in glioma cells remains to be clarified.

In contrast to the effect of a MEK1 inhibitor that did not affect apoptosis induction, a caspase inhibitor was found to block both cell cycle progression and apoptosis and also to inhibit the ERK phosphorylation induced by Fas. This indicates that the Fas-mediated caspase activation in glioma cells plays two independent roles for opposite cellular responses; one is to induce apoptosis and another is to act upstream of the MEK/ERK pathway that is linked to cell cycle progression. In this context, it is noteworthy that the Fas-mediated p38/JNK activation was also shown to be blocked by caspase inhibitors (10–12, 16, 33) and that caspase-mediated proteolytic cleavage of several molecules that potentially activate MAPKs have been reported, including PAK2 (15), MAP kinase kinase 6b (33), and MAPK/ERK kinase kinase 1 (12). In SHEP cells, a dominant-negative form of Ras inhibited the Fas-mediated ERK and JNK activation (14). Identification of the 2D-sensitive caspase(s) acting upstream of ERK in glioma cells awaits further investigation, and apparently the cell type-specific regulation of the Ras pathway downstream of Fas is to be the target of further research.

Regarding the differential roles of MAPK subfamilies in regulating growth or death, our results are complementary with those by Xie et al. (34) who have shown that withdrawal of nerve growth factor from rat PC-12 pheochromocytoma cells resulted in sustained activation of JNK and p38 with concurrent inhibition of ERK, which was associated with apoptosis induction. Similarly, it has been shown that, downstream of the T-cell receptor signaling in thymocytes, the MEK1-ERK pathway was linked to positive selection by promoting thymocyte survival and proliferation, whereas the MAP kinase kinase 6-p38 pathway was involved in negative selection by promoting apoptosis (35). Among the Fas receptor-binding molecules, FADD plays a critical role in initiating cell death process by triggering the caspase cascade, but paradoxically, several lines of evidence have suggested that it may regulate cell proliferation as well: FADD-deficient T cells (36) and dominant-negative FADD-expressing T cells did not proliferate normally in response to mitogens (37). In light of this knowledge, our findings are unique in that both cell death and cell growth were induced simultaneously in response to signaling via a single receptor. The glioma cells may thus provide a unique cell culture system for elucidating molecular switch mechanism(s) that determine cell death or growth downstream of Fas.

Because little Fas and Fasl mRNA could be detected in the normal brain (38), it was previously thought that the Fas-FasL system might not be operative in the central nervous system. However, subsequent studies have revealed that this system is actively operative in the central nerve system in some pathological conditions. Activated microglia expressed Fasl, which in turn may induce Fas-expressing oligodendrocyte death in multiple sclerosis (39). As shown in this article and reported by others, some glioma cell lines as well as primary astrocytic brain tumors express Fas and/or Fasl (8, 22, 40). Our present study extends these observations and provides a strong implication that gliomas use the endogenous Fas-Fasl interaction for proliferation. Fasl expression in tumors has been implicated as a means to evade antitumor immune response (5, 6). Regarding the pathophysiological role of Fas/Fasl expression in tumorgenesis, our present study may thus provide an important implication that some tumors may use this system for progression by promoting its own growth as an autocrine growth factor.

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