Growth-inhibitory Effect of a Streptococcal Antitumor Glycoprotein on Human
Epidermoid Carcinoma A431 Cells: Involvement of Dephosphorylation of
Epidermal Growth Factor Receptor

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ABSTRACT

An antitumor glycoprotein [streptococcal acidic glycoprotein (SAGP)] purified from an extract of Streptococcus pyogenes inhibited the growth of human epidermoid carcinoma A431 cells expressing epidermal growth factor receptor (EGFR) in a time- and a concentration-dependent manner. The antiproliferative effect of SAGP was diminished by preincubating the cells with pertussis toxin and by coadministration of sodium orthovanadate, an inhibitor of protein tyrosine phosphatases (PTPases). Western blot analysis showed that the immunoreactivity of a Mr 170,000 band of cell lysate to antiphosphotyrosine antibody was reduced by SAGP, and the effect was abolished by sodium orthovanadate. The phosphotyrosine level of the precipitant with anti-EGFR antibody was reduced by SAGP, which was abolished by preincubation with pertussis toxin or by a coadministration with sodium orthovanadate. The PTPase activity transiently increased in the lysate of cells incubated with SAGP and was inhibitable by sodium orthovanadate. Additionally, preincubation of serum-starved A431 cells with SAGP decreased the epidermal growth factor-induced tyrosine phosphorylation of EGFR, and the effect of SAGP was sodium orthovanadate sensitive. These findings indicate that dephosphorylation of the Mr 170,000 EGFR by activation of PTPase(s) may be responsible in part for the antiproliferative effect of SAGP on A431 cells.

INTRODUCTION

The Streptococcus pyogenes Su strain, from which immunomodulating agent OK-432 (1) was prepared, provides a novel antitumor SAGP as reported previously (2,3). SAGP is a glycoprotein with an apparent molecular weight of 140,000–150,000 that consists of identical subunits with a molecular weight of 48,000. This protein has been shown to prolong the life span of mice inoculated i.p. with Ehrlich ascite carcinoma cells (2) or murine Meth A cells (4). The life-prolonging effect of SAGP on the mice inoculated with Ehrlich ascite carcinoma cells or Meth A cells is known to be reduced by immunosuppression with X-ray irradiation or antimacrophage agent carrageenan injection, indicating that the antitumor effect of SAGP depends in part on the activation of the host immune system. Indeed, an in vitro assay revealed considerable cytostatic activity of spleen cells (carrageenan sensitive and/or asialo GM1 positive) from Meth A-inoculated and SAGP-injected mice against Meth A cells (3, 4).

On the other hand, the direct inhibitory effect of SAGP on cell proliferation has been reported on transformed hamster embryonic lung cells (2), murine leukemia L1210 cells (5), and Meth A cells (6, 7) in culture. Sulphhydryl groups on SAGP appear to be essential for the expression of SAGP activity because the cell growth-inhibitory effect on Meth A cells was reduced by sulfhydryl-oxidizing agents such as cystamine and 5,5'-dithio-bis(2-nitrobenzoic acid) (6). The activation of IAP-sensitive GTP-binding protein (G protein) is known to be required for expression of the antiproliferative effect of SAGP, and the inhibition of nucleic acid synthesis may contribute to the direct effect of SAGP (7). Although the cAMP level in Meth A cells exposed to SAGP was reduced slightly (7), it was unlikely that the reduced cAMP levels contributed to the SAGP-induced cell growth inhibition because dibutyryl-cAMP or a cAMP phosphodiesterase inhibitor could not reverse the SAGP activity.3

Our recent study with Meth A cells revealed that the antiproliferative effect of SAGP was diminished by sodium orthovanadate, an inhibitor of PTPases, but not by a serine/threonine phosphatase inhibitor (8). Western blot analysis revealed that immunoreactivity of a Mr 170,000 cellular protein to antiphosphotyrosine Ab was reduced in the cells incubated with SAGP, and the effect was abolished by sodium orthovanadate (8). These findings led us to a working hypothesis that SAGP binds to an IAP-sensitive G protein-coupled unknown receptor and activates PTPases, resulting in a decrease of tyrosine phosphorylation of the Mr 170,000 cellular protein. It was suggested that the dephosphorylated Mr 170,000 cellular protein may be a growth factor receptor because some studies have demonstrated that stimulation of G protein-coupled receptor activates intracellular PTPases (9, 10) and that PTPases directed to growth factor receptors inhibit the autophosphorylation of growth factor receptors (11–13). Therefore, we intended to identify the Mr 170,000 protein, to confirm the above-mentioned hypothesis, and to further elucidate the mechanism of the antiproliferative action of SAGP. Although most previous studies have been performed with Meth A cells, we used human epidermoid carcinoma A431 cells here because this cell line is known to overexpress EGFR, and a variety of immunological tools such as Abs to the cell growth signaling components are commercially available.

Similar to the results obtained from Meth A cells, the growth of A431 cells was inhibited by SAGP in a concentration- and a time-dependent manner. The antiproliferative effect of SAGP was reduced by preincubating the cells with IAP or by coadministration of sodium orthovanadate. In this study, we identified the Mr 170,000 protein of A431 cells as an EGFR and showed that stimulation of the IAP-sensitive G protein-coupled receptor by SAGP caused dephosphorylation of the EGFR via an activation of PTPases, leading to the inhibition of cell growth.

MATERIALS AND METHODS

Preparation of SAGP. SAGP was prepared as described previously (2, 7). Tumor Cell Lines. The human epidermoid carcinoma A431 cells were kindly supplied by Prof. Katsumi Nishikawa (Second Department of Biochemistry, Kanazawa Medical University) and cultured in DMEM containing 10% heat-inactivated FBS, 2 mM l-glutamine, 12.7 mM HEPES, 0.12% sodium bicarbonate, 100 units/ml penicillin G, and 100 μg/ml streptomycin at 37°C in humidified air containing 5% CO₂. Cells were seeded at 3 × 10³ cells/plate in

3 Unpublished data.
10-cm diameter plastic culture dishes and passaged every 3–4 days. The doubling time of A431 cells at densities of 3–30 x 10^4 cells/ml was 22–24 h.

**Cell Growth Assay.** A431 cells were seeded at 3 x 10^4 cells/ml in 35-mm-diameter culture dishes (2.5 ml/plate). After an overnight attachment phase, the DMEM containing 10% FBS was refreshed, and SAGP (0.03–1.0 µg/ml) was added. After the specified times of incubation, the number of cells was determined by trypsin blue dye exclusion after trypsinization. The cell growth rate (percentage of control) was expressed as the percentage of the number of the cells in the wells with SAGP: the number of cells in the control wells.

**Stimulation and Extraction of Cells.** A431 cells were plated at 3 x 10^4 cells/ml in 100-mm-diameter culture plates (10 ml/plate). After an overnight attachment phase, the DMEM containing 10% FBS was refreshed, and the cells were incubated with or without SAGP (0.1 and 0.3 µg/ml protein/ml) for 48 h. The stimulated cells were washed twice with PBS and lysed in 1 ml of radioimmunoprecipitation assay buffer (PBS containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, and 57 µg/ml aprotinin) by repeated passage through a 23-gauge needle. The lysates were centrifuged at 12,500 x g for 20 min at 4°C to remove insoluble material. After protein concentrations were determined with Bio-Rad protein assay reagent (Bio-Rad Laboratories), the cell lysate was used to remove insoluble material. The results from duplicate wells for each sample were compared with a standard curve with CD45 (Calbiochem-Novabiochem) and expressed as phosphate released/min/µg protein.

**Assay for EGF-induced EGFR Signaling.** To ascertain the effect of SAGP on the EGF-induced EGFR phosphorylation and downstream elements involved in EGF-induced mitogenic signaling, A431 cells were incubated in DMEM containing 10% FBS for 2 days and then incubated for 24 h in DMEM without FBS. The serum-starved A431 cells were preincubated with or without SAGP for the indicated time at 37°C and then stimulated with 5 ng/ml EGF (Sigma Chemical Co.) for 5 min at 37°C and lysed. Tyrosine phosphorylation of EGFR was assayed by Western blotting of immunoprecipitated EGFR with antiphosphotyrosine Ab (clone 4G10) as described above. To look at the effect of SAGP on p42/44 MAPK, the total cell lysate (25 µg of protein) was resolved by SDS-PAGE (10% gels). A Western blot was probed with an Ab that recognizes phospho-p42/44 MAPK (New England Biolabs). To control the amount of p42/44 MAPK, the blot was stripped and reprobed with a goat polyclonal anti-p42 MAPK Ab (Santa Cruz Biotechnology) that reacts with p42 MAPK and, to a lesser extent, with p44 MAPK. Antimouse or antigo IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology) was used as the secondary Ab, and proteins were visualized with ECL-PLUS Western blotting detection reagents (Amersham Pharmacia Biotech).

### RESULTS

**Growth-inhibitory Action of SAGP on A431 Cells.** SAGP inhibited the growth of A431 cells in a concentration- and time-dependent manner. The mean ± SE of six cultures from three separate experiments. a, the time course of the effect of SAGP on A431 cell growth. A431 cells (3 x 10^4 cells/ml) were incubated in the absence or presence of SAGP for 3 days. The values are the mean ± SE of six cultures from three separate experiments. b, the time course of the effect of SAGP on A431 cell growth. A431 cells (3 x 10^4 cells/ml) were incubated in the absence or presence of SAGP for 3 days. SAGP (0.1 or 0.3 µg/ml) was added to the cultures on day 0. The values are the mean ± SE of six cultures from two separate experiments.

Fig. 1. a, growth-inhibitory effect of SAGP on A431 cells in culture. The cells (3 x 10^6 cells/ml) were incubated in the absence or presence of SAGP for 3 days. The values are the mean ± SE of six cultures from three separate experiments. b, the time course of the effect of SAGP on A431 cell growth. A431 cells (3 x 10^4 cells/ml) were incubated in the absence or presence of SAGP for 3 days. SAGP (0.1 or 0.3 µg/ml) was added to the cultures on day 0. The values are the mean ± SE of four cultures from two separate experiments.
manner (Fig. 1, a and b). The median inhibitory concentration (IC\(_{50}\)) of SAGP on the growth of A431 cells after 3 days of incubation was 0.08 μg protein/ml. The growth inhibition rate increased gradually up to day 3.

Effects of IAP, Sodium Orthovanadate, and Catalase on the Growth-inhibitory Effect of SAGP. Similar to the result obtained with the Meth A cell line (7), pretreatment of A431 cells with 100 ng/ml IAP (Seikagaku, Tokyo, Japan) for 6–16 h decreased the antiproliferative effect of SAGP (Fig. 2). Sodium orthovanadate, an inhibitor of PTPases, significantly diminished the growth-inhibitory activity of SAGP on A431 cells, although sodium orthovanadate by itself had a toxic effect on the cells in a concentration-dependent manner (Fig. 3). Sodium fluoride (1 mM), a serine/threonine phosphatase inhibitor, did not affect the growth-inhibitory action of SAGP (data not shown). However, the growth-inhibitory effect of SAGP was augmented by coadministration of catalase (Fig. 4). The augmentation by catalase has also been observed in Meth A cells.3

Tyrosine Phosphorylation of Cellular Proteins in A431 Cells. Western blot analysis on the cell lysate using antiphosphotyrosine Ab revealed that a 48-h exposure of cells to SAGP (0.1 and 0.3 μg protein/ml) caused a decrease in phosphotyrosine immunoreactivity at \( M_r 170,000 \) cellular protein. No differences in the phosphotyrosine level between samples from SAGP-treated or PBS-treated cells were seen in the presence of 30 μM sodium orthovanadate (Fig. 5). This suggests that the reduction in the phosphotyrosine level of \( M_r 170,000 \) protein may be due to the activation of PTPases by SAGP.

Tyrosine Phosphorylation of Immunoprecipitated EGFR. The phosphotyrosine level of immunoprecipitated EGFR was decreased in the cells incubated with SAGP for 48 h in the presence of 10% FBS, whereas the content of EGFR immunoprecipitated from an equal amount of cell lysate was not changed by incubating the cells with SAGP for 48 h (Fig. 6a). The effect of SAGP was abolished by coadministration of 30 μM sodium orthovanadate, although sodium orthovanadate itself stimulated basal tyrosine phosphorylation (Fig. 6b). Pretreatment of cells with IAP (100 ng/ml) for 6–16 h diminished the inhibitory effect of SAGP on tyrosine phosphorylation of EGFR (Fig. 6b). Fig. 6c shows the average (percentage of control) signal intensity on scanning densitometry, demonstrating that the SAGP-induced dephosphorylation of EGFR was abolished by sodium orthovanadate and inhibited in part by IAP, although quantification of the signal by scanning densitometry is nonlinear and therefore generates numbers that cannot be strictly interpreted. Neither IAP nor sodium orthovanadate alone affected EGFR protein levels (data not shown).

PTPase Activity. When A431 cells were exposed to SAGP (0.3 μg protein/ml) for various times in the presence of 10% FBS, significant increases in PTPase activity were detected 1 and 2 min after the addition of SAGP. No differences in the PTPase activity between control and SAGP-stimulated cells were seen at 5, 10, 30, and 60 min of incubation, although control PTPase activity tended to increase gradually and at 60 min reached 150% of that at 1 min (Fig. 7a). The SAGP-induced stimulation of PTPase activity at 2 min was inhibited by coadministration of 30 μM sodium orthovanadate (Fig. 7b).

Effect of SAGP on EGF-stimulated Mitogenic Signaling. Serum-starved A431 cells were incubated with or without SAGP (0.1 and 0.3 μg protein/ml) for 16 h before stimulation with EGF (5 ng/ml, 5 min), and cells were lysed. The immunoprecipitated EGFR was resolved by SDS-PAGE (7.5% gels), and a Western blot was probed with an antiphosphotyrosine Ab (clone 4G10). As shown in Fig. 8a,
EGF-induced tyrosine phosphorylation of EGFR was diminished in the lysate of cells preincubated with SAGP. The effect was not observed in the extract of cells preincubated with SAGP and sodium orthovanadate. A kinetic analysis of the SAGP-induced EGFR dephosphorylation revealed that a decrease in EGFR phosphorylation was observed between 30 and 60 min of cell preincubation with SAGP (Fig. 8b).

Furthermore, we examined the effect of SAGP on EGF-induced p42/44 MAPK activation by Western blot analysis using an Ab to phosphorylated p42/44 MAPK because trans-inactivation of the EGFR by G protein-coupled receptors, independent of the MAPK pathway, has been demonstrated for bradykinin in A431 cells by Graness et al. (14). Fig. 9, a and c, shows that the pretreatment of cells with SAGP (0.3 μg protein/ml) for 30 min and 1 h caused a decrease in EGF-induced p42/44 MAPK phosphorylation, whereas the protein levels are equal. The inhibitory effect of SAGP on p42/44 MAPK phosphorylation at 30 min was concentration dependent (Fig. 9b).

DISCUSSION

In previous studies, we demonstrated that the antiproliferative effect of SAGP on murine Meth A cells was diminished by incubating the cells with IAP and that SAGP actually augmented the activity of IAP-sensitive G protein as assessed by IAP-catalyzed ADP ribosylation (7). In addition, the Meth A cell growth-inhibitory effect of SAGP was diminished by coadministration of sodium orthovanadate, an inhibitor of PTPases. Western blot analysis in Meth A cell lysate with antiphosphotyrosine Ab demonstrated that phosphotyrosine content of a M₉ 170,000 cellular protein was decreased by incubating the cells with SAGP and that the decrease was inhibited by coadministration of sodium orthovanadate, suggesting the involvement of activation of PTPases.

The present study shows that SAGP inhibited proliferation of A431 cells, human epidermoid carcinoma cells overexpressing EGFR, in a concentration- and a time-dependent manner (Fig. 1, a and b). The growth-inhibitory effect of SAGP on A431 cells was diminished by preincubating the cells with IAP (Fig. 2). The activity of SAGP was inhibited in part by coadministration of sodium orthovanadate (Fig. 3). Western blot analysis on A431 cell lysate revealed that the immunoreactivity of a M₉ 170,000 cellular protein to antiphosphotyrosine Ab was decreased by incubating the cells with SAGP. The effect was abolished by coadministration of sodium orthovanadate (Fig. 5). Thus, the involvement of both IAP-sensitive G protein and inhibition of
tyrosine phosphorylation of the M, 170,000 cellular protein by PTPases in a tumor cell growth-inhibitory effect of SAGP was confirmed in the A431 human epidermoid cell line.

Then, to determine the M, 170,000 cellular protein reactive to antiphosphotyrosine Ab, we investigated the effect of SAGP on the tyrosine phosphorylation of EGFR in A431 cells. The phosphotyrosine content of the precipitant with anti-EGFR Ab was decreased in the extract of cells incubated with SAGP, although the amounts of EGFR were equal (Fig. 6a). The SAGP-induced inhibitory effect on EGFR phosphorylation was diminished by incubating the cells with SAGP and sodium orthovanadate or by preincubating the cells with IAP (Fig. 6, b and c). These findings indicate that the dephosphorylated M, 170,000 protein corresponds to EGFR and also indicate that the dephosphorylation of the EGFR is mediated by sodium orthovanadate-sensitive PTPases and IAP-sensitive G protein.

To clarify whether SAGP could activate PTPases, we next measured the PTPase activity in the cell lysate using a nonradioactive assay kit. When the cells were exposed to SAGP in the presence of 10% FBS, the PTPase activity in cell lysate increased significantly as early as 1–2 min after the addition of SAGP (Fig. 7a). The activation of PTPase by SAGP at 2 min was abolished by coadministration of sodium orthovanadate (Fig. 7b). The reason for the discrepancy in the kinetics between the EGFR dephosphorylation and the PTPase activation by SAGP is unclear, but it might be due to the experimental conditions for the PTPase assay. In this assay, we measured the total cellular and membrane-bound PTPases, but we did not measure the specific PTPase being activated by SAGP and associating with the phosphorylated EGFR. Net SAGP-induced PTPase activation might be detected only 1 and 2 min after the cells were stimulated with SAGP in the presence of 10% FBS because growth factors such as insulin and platelet-derived growth factors in FBS would activate cellular PTPases. Some reports demonstrated that PTPase plays a positive role in growth factor-stimulated cell proliferation (15, 16).

With regard to the activation of PTPase by SAGP, we observed in the cell growth assay that the antiproliferative effect of SAGP on A431 cells was augmented by a coadministration of catalase as shown in Fig. 4. Bae et al. (17) reported that stimulation of A431 cells with EGF resulted in a transient increase in the intracellular concentration of reactive oxygen species (predominantly H₂O₂) and that the effect of EGF was abolished by the incorporation of catalase into the cells. In addition, Lee et al. (18) demonstrated that H₂O₂ inactivated recombinant PTP1B in vitro by oxidizing its catalytic site cysteine, suggesting the concurrent inhibition of PTPases by H₂O₂ produced in response to EGF. The present finding that the SAGP activity was augmented by catalase may be additional evidence suggesting the involvement of the activation of PTPases in antiproliferative signaling by SAGP.

The identity of activated PTPases is currently unknown. It has been shown that the cellular PTPases play a prominent role in growth factor-mediated signal transduction (19–21). The list of PTPases shown to possess the capacity for interaction with growth factor

Fig. 7. The effect of SAGP on PTPase activity. A431 cells were incubated with SAGP or PBS for various times. The cell lysates were prepared, and PTPase activity was assayed as described in "Materials and Methods." a, effect of incubation time on PTPase activity. The results (mean ± SE) are expressed as a percentage of basal PTPase activity obtained from PBS-incubated cells at 1 min. *P < 0.05 versus each control (without SAGP) by unpaired Student's t test. PTPase activity in PBS-incubated cells at 1 min averaged 0.021 ± 0.002 nmol phosphate/min/μg protein. b, effect of sodium orthovanadate on SAGP-induced PTPase activation at 2 min. The results (mean ± SE) are expressed as a percentage of basal PTPase activity obtained from PBS-incubated cells at 2 min. Statistical analyses of the variables of each group were performed by one-way ANOVA followed by Fisher’s PLSD (*, P < 0.05 versus control cells without SAGP). PTPase activity in PBS-incubated cells at 2 min averaged 0.024 ± 0.005 nmol phosphate/min/μg protein.

Fig. 8. Effect of SAGP on EGF-stimulated tyrosine phosphorylation of EGFR. a, serum-starved A431 cells were preincubated with SAGP (0.1 and 0.3 μg protein/ml) in the presence or absence of 30 μM sodium orthovanadate for 16 h and then stimulated for 5 min with 5 ng/ml EGF. b, serum-starved A431 cells were preincubated with SAGP (0.3 μg protein/ml) for 16 h, 1 h, 30 min, 15 min, and 5 min and then stimulated for 5 min with 5 ng/ml EGF. After stimulation with EGF, cells were lysed, and cell lysates were immunoprecipitated with an anti-EGFR Ab. Immunoprecipitates were analyzed by SDS-PAGE (7.5% gels) followed by Western blot analysis with an antiphosphotyrosine Ab (clone 4G10). Similar results were obtained from three (a) or two (b) independent experiments.

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SAGP (0.3 μg protein/ml) or preincubated with SAGP (0.1–1.0 μg protein/ml) for 30 min (a), respectively, and then stimulated with EGF (5 ng/ml, 5 min). After stimulation with EGF, cells were lysed, and the cell lysates (25 μg protein/ml) were resolved by SDS-PAGE (10% gels) followed by Western blotting with an Ab to phospho-p42 MAPK. The membrane was reprobed with an Ab to p42 MAPK as described in Materials and Methods. c shows the relative phosphorylation level of p42/44 MAPK to its protein (percentage of control). Statistical analyses of the variables from three independent experiments (mean ± SE) were performed by one-way ANOVA followed by Fisher’s PLSD (*, P < 0.05 versus control).

Fig. 9. Effect of SAGP on EGF-stimulated p42/44 MAPK activation. Serum-starved A431 cells were preincubated with SAGP (0.3 μg protein/ml) for the indicated time (a) or preincubated with SAGP (0.1–1.0 μg protein/ml) for 30 min (b), respectively, and then stimulated with EGF (5 ng/ml, 5 min). After stimulation with EGF, cells were lysed, and the cell lysates (25 μg protein/ml) were resolved by SDS-PAGE (10% gels) followed by Western blotting with an Ab to phospho-p42/44 MAPK. The membrane was reprobed with an Ab to p42 MAPK as described in “Materials and Methods.” c shows the relative phosphorylation level of p42/44 MAPK to its protein (percentage of control). Statistical analyses of the variables from three independent experiments (mean ± SE) were performed by one-way ANOVA followed by Fisher’s PLSD (*, P < 0.05 versus control).

All of the above-mentioned findings combine to suggest a model for signal transduction of the antiproliferative effects of SAGP, as shown in Fig. 10. Namely, SAGP binds to an IAP-sensitive G protein-coupled receptor that has yet to be identified and activates PTases followed by dephosphorylation of EGFR, resulting in inhibition of cell proliferation, probably via inhibition of p42/44 MAPK. However, we cannot conclude that the antiproliferative action of SAGP is achieved solely through PTase activation because SAGP activity is incompletely abolished by sodium orthovanadate or IAP.

With regard to the antiproliferative mechanism, interest has focused on the antitumor effect of the neuropeptide somatostatin and its analogues (30–33). Stimulation of G protein-coupled receptor by somatostatin (34) has been shown to exhibit an antiproliferative effect on tumor cells via an activation of PTases (10, 35–38). Recent studies demonstrated that the activated PTases dephosphorylate the growth factor receptors, resulting in cell growth inhibition (13, 19, 39). It has also been shown that the antiproliferative effect of TNF is associated with alterations in specific PTases (11, 40). Recently, Perez et al. (41) demonstrated that TNF induced a translocation of PTP1B-related proteins in tumor cells that interacts with EGFR, resulting in growth inhibition. Guo et al. (21) also showed that a

![Fig. 10. A suggested model for signal transduction of the antiproliferative effect of SAGP. Tyr-P, phosphotyrosine.](image-url)
PTPase activated by TNF inhibits activation of vascular endothelial growth factor receptor KDR and vascular endothelial growth factor-induced endothelial cell proliferation. The present findings may support the concept that activation of growth factor receptor-directed PTPases could contribute to a mechanism for novel antiproliferative agents.

Furthermore, SAGP may also provide a tool to examine a cross-regulation between stimulation of G protein-coupled receptor and growth factor receptor signaling.

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