Jpn. J. Cancer Res. 87, 459–465, May 1996

Oncogenic Collaboration of the Cyclin D1 (PRAD1, *bcl*-1) Gene with a Mutated p53 and an Activated *ras* Oncogene in Neoplastic Transformation

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Cyclin D1 is one of the key regulators in G1 progression in the cell cycle and is also a candidate oncogene (termed PRAD1 or *bcl*-1) in several types of human tumors. We report a collaboration of the cyclin D1 gene with *ras* and a mutated form of p53 (p53-mt) in neoplastic transformation. Transfection of cyclin D1 alone or in combination with *ras* or with p53-mt was not sufficient for focus formation of rat embryonic fibroblasts. However, focus formation induced by co-transfection of *ras* and p53-mt was enhanced in the presence of the cyclin D1-expression plasmid. Co-transfection of *ras*- and p53-mt-transformants with the cyclin D1-expression plasmid resulted in reduced serum dependency *in vitro*. Furthermore, the transformants expressing exogenous cyclin D1 grew faster than those without the cyclin D1 plasmid when injected into nude mice. These observations strengthen the significance of cyclin D1 overexpression through gene rearrangement or gene amplification observed in human tumors as a step in multistep oncogenesis; deregulated expression of cyclin D1 may reduce the requirement for growth factors and may stimulate *in vivo* growth.

Key words: Cyclin D1 — Transfection experiment — Transformation — p53 — *ras*

Cyclins regulate cell cycle progression by controlling the activities of cyclin-dependent kinases at various checkpoints.[1,2] Deregulated expression of cyclins is likely, therefore, to have a great impact on cell growth and proliferation. In fact, the cyclin D1 gene is strongly implicated in the development of human tumors, including parathyroid adenomas (as PRAD1),[3–5] B-cell lineage tumors with t(11;14)(q13;q32) (as *bcl*-1),[6,7] breast[8,9] and esophageal[10] cancers, squamous cell cancers of head and neck and others.[11,12] The cyclin D1 gene is overexpressed through gene rearrangement or gene amplification in tumor cells. However, the oncogenic role of cyclin D1 overexpression in tumors is largely unknown.

We used rat embryonic fibroblasts for transfection experiments to analyze the oncogenic significance of cyclin D1 gene overexpression in an experimental system. Cyclin D1 alone proved to be inactive in focus formation. However, forced expression of cyclin D1 further facilitated focus formation induced by co-transfection of *ras* and p53 mutant oncogenes, and also had an impact on cell growth *in vitro* and *in vivo*.

MATERIALS AND METHODS

Plasmids Expression plasmids for a mutated form of mouse p53 gene (p53-KH215, p53-mt)[13] and for an activated allele of human Ha-*ras* (pucEJ, *ras*)[14] were generous gifts of Dr. P. W. Hinds, Harvard Medical School and Dr. S. Dowdy, Whitehead Institute, Boston. PRAD1/cyclin D1-expression plasmid, pSVD1, was constructed by replacing the c-*jun* insert of pSV2humjun,[15] which has an SV40 early promoter and SV40 splice-poly A signals, and was provided by Dr. T. D. Halazonetis, Merck Sharp & Dohme Research Laboratories, West Point, PA, with 1.1-kb *Eco*RI-*Hin*dIII fragment derived from pPl-8/λPl-4.[5] pSVD1 has the full coding sequence and truncated 3′ untranslated region of the human PRAD1/cyclin D1 gene. The control plasmid, pSV0, was constructed by removing the insert of pSV2humjun. The neo gene, pcDneo,[16] was a generous gift from Dr. H. Okayama, University of Tokyo, Japan.

Preparation of rat embryonic fibroblasts (REFs) Fetuses were removed from 14-day pregnant Wistar rats (Tokyo Laboratory Animals Science Co., Ltd., Tokyo). The fetus was decapitated and the viscera were removed. The remainder was minced, then cells were trypsinized for 30 min and washed with Dulbecco's modified Eagle's medium (D-MEM, GibcoBRL Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, BioWhittaker, Walkersville, Maryland) and kanamycin 60 μg/ml (Meiji Seika Kaisha, Ltd., Tokyo) (complete medium). Cells were resuspended in complete medium, and aggregates and tissue debris were sedimented for 1 min. Floating separate cells were transferred to culture dishes and incubated in complete medium at 37°C in a humidified atmosphere with 5%

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CO_2 for 2 days (until confluency was achieved) then stored in liquid nitrogen in 5% dimethyl sulfoxide/complete medium until DNA transfection experiments.

DNA transfection and focus formation assay DNA transfection was performed mostly according to Chen and Okayama.[16] In brief, frozen REFs or COS7 cells, which were provided by Dr. M. Matsuoka, Institute of Medical Science, University of Tokyo, were thawed, plated ($3-5\times10^5$/60-mm dish) and incubated overnight at 37°C under 5% CO_2. DNA (20 μg) was mixed with 0.25 *M* $CaCl_2$ 250 μl and 2× BES-buffered saline 250 μl and left at room temperature for 10 to 20 min. The DNA mixture was poured into the culture and it was incubated at 35–37°C under 3–4% CO_2 for 14 to 18 h. The cells were washed with phosphate-buffered saline (PBS, Dulbecco's PBS, Nissui Pharmaceutical Co., Ltd., Tokyo) twice and incubated overnight in complete medium at 37°C under 5% CO_2. Trypsinization of the REFs after two PBS washes yielded about $3-5\times10^5$ cells, which were divided into two portions, each of which was cultured in a 100-mm dish. G418 (400 μg/ml, GibcoBRL) was added to one of them the next day to confirm successful DNA transfection. The medium was replaced every 3 to 4 days thereafter. Foci were detectable 4 to 5 days after trypsinization. The cells were dried, fixed with methanol and stained in Giemsa solution 10 to 12 days after trypsinization. The foci with a diameter of 3 mm or more were counted.

Immunoblot analysis Cells were lysed with 1× sample buffer [TrisCl 60 m*M*, sodium dodecyl sulfate (SDS) 2%, dithiothreitol 0.1 *M*, pH 6.8] after two PBS washes, boiled for 5 min and stored at −20°C until analysis. Protein concentrations were determined using BCA protein assay reagent (Pierce, Rockford, IL) with bovine serum albumin as a standard, as described elsewhere.[17] Protein was separated on 10% SDS-polyacrylamide gel and blotted onto nitrocellulose membrane using semi-dry electrophoretic transfer as described.[18] Blocking was performed in 5% nonfat milk/PBS for 2 h and the blocked membrane was incubated overnight with murine monoclonal antibody (HD63) against PRAD1/cyclin D1 protein, which was generated as described[19] and used at 1:5 dilution. Alkaline phosphatase-conjugated rabbit anti-mouse IgG1 antibody (Zymed Laboratories, Inc., San Francisco, CA; 1:1000 dilution) was used as the secondary antibody and cyclin D1 protein was visualized by enzyme-catalyzed color reaction as described by the manufacturer (Boehringer Mannheim Biochemica, Mannheim, Germany). Human cyclin D1 protein was synthesized in bacteria as described[5] and used as a positive control.

Northern blot analysis RNA preparation and Northern blot analysis were performed as previously described.[20] Each aliquot (10 μg) of RNA was separated on a formaldehyde-agarose gel, blotted onto nitrocellulose membrane, and hybridized with the random-primed ^{32}P-labeled cyclin D1 probe, the *Eco*RI 1.4-kb insert of pPl-8/λPl-4.[5] The blot was autoradiographed at −80°C with an intensifying screen. The same membrane was rehybridized with a β-actin probe as a control.

Assay for serum dependency Transformants were plated at 4×10^4 per well ($2\times10^4/cm^2$) in 24-well plates and were incubated in complete medium for a day. One day after replacing the medium with D-MEM containing the indicated concentration of FBS, [^3H]thymidine (0.5 μCi/ml, Amersham, Buckinghamshire, England) was added and [^3H]thymidine incorporation during 24 h was measured according to Danielpour *et al.*[21]

Assay for tumorigenicity Five-week-old male athymic mice were purchased from Clea Japan Inc. (Tokyo) and were maintained in sterilized cages. Tumorigenicity was studied by inoculating the indicated amount of cells subcutaneously into the right flank of nude mice. Tumor size was measured weekly and was calculated by use of the following formula: tumor volume (ml) $= a\times b^2/2$, where a is the length (cm) and b is the width (cm) of the tumor. The animals were treated in accordance with Chugai Pharmaceutical's ethical guidelines of animal care, handling and termination, and the protocols were approved by the Animal Care Committee of the institution.

RESULTS

In order to examine the cyclin D1 gene for possible oncogenic functions, we generated a cyclin D1-expression plasmid, pSVD1. COS7 cells were chosen to examine pSVD1 for the capacity to express human cyclin D1 protein because COS7 cells themselves did not express any detectable amount of cyclin D1 protein. Whole cell lysates were harvested 3 days after transfection with the plasmid by the calcium phosphate method as described under "Materials and Methods." After transfection with pSVD1, cells expressed exogenous cyclin D1 protein, which migrated to the same position as did bacterially expressed cyclin D1 protein (Fig. 1).

Focus formation assays using rodent cells have been utilized to analyze the functions of various tumor-related genes. Two different types of oncogenes are required to transform normal REFs, so that they can generate foci on a monolayer of normal cells. While transfection of either *ras* or p53-mt did not significantly induce foci, transfection of both *ras* and p53-mt led to the appearance of multiple foci (Fig. 2, A and B), as reported previously.[22, 23] Additional transfection of pSVD1 increased the efficiency of focus formation induced by *ras* and p53-mt, although transfection of pSVD1 alone or in combination with *ras* or p53-mt had no effect. The foci appeared to grow larger in the presence of pSVD1 than those induced by *ras* plus p53-mt.

To analyze further the significance of addition of the cyclin D1-expression plasmid, we examined the transformants separately derived from each focus to evaluate the impact of pSVD1 on the growth characteristics *in vitro* and *in vivo*. Five well-separated foci generated after transfection with *ras* and p53-mt (pSVD1-minus) or with *ras*, p53-mt and pSVD1 (pSVD1-plus) were randomly trypsinized with cloning cylinders, and cell lines were established and passaged for more than 3 months. By Northern blot analysis, transcripts derived from transfected pSVD1 were easily detected as aberrant messages in the pSVD1-plus transformants (Fig. 3), except for one cell line which was excluded from subsequent analysis. As evaluated by immunoblot analysis, the amounts of cyclin D1 protein expressed in the transformants were highly variable, and the average amount of expressed cyclin D1 protein in pSVD1-plus transformants was slightly, but not significantly, larger than that of pSVD1-minus cells (data not shown).

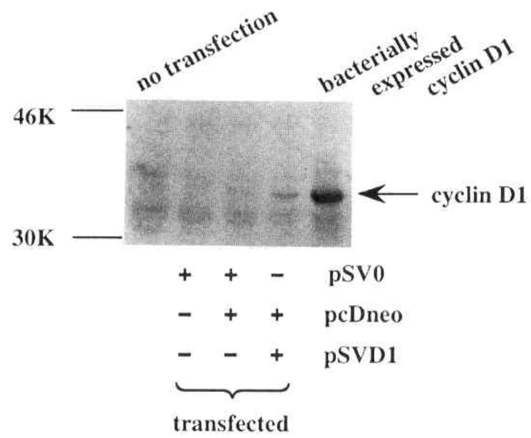

Fig. 1. Expression of human cyclin D1 in COS7 cells after transfection with pSVD1 plasmid. COS7 cells were transfected with the indicated plasmids (10 μg each): pSV0, control plasmid; pcDneo, neo gene plasmid; pSVD1, human cyclin D1-expression plasmid. Whole cell lysates (50 μg protein/lane) were immunoblotted with anti-human cyclin D1 monoclonal antibody, HD63. Cyclin D1 protein was visualized by enzyme-catalyzed color development. Cyclin D1 (arrow) was not detectable in COS7 cells with no transfection. Bacterially expressed cyclin D1 protein served as a control. Molecular markers are shown on the left.

The *in vitro* growth of these cell lines was measured by counting trypan blue-excluding cells with a hemocytometer. The doubling times of the cells at the log phase were exactly the same for pSVD1-plus ($n=4$) and minus ($n=5$) cells (mean±SD: 14.5±4.1 h and 14.4±2.3 h, respectively). However, the serum dependency of these cell lines was different with respect to the presence of pSVD 1. In culture medium containing 2% FBS, reduction of thymidine incorporation by the pSVD1-plus transformants was significantly less than that by the minus cells (Fig. 4), although the pSVD1-plus cells were still serum-dependent as shown at 0.2% FBS/medium.

To investigate further the impact of the cyclin D1-expression plasmid, equal amounts of the established

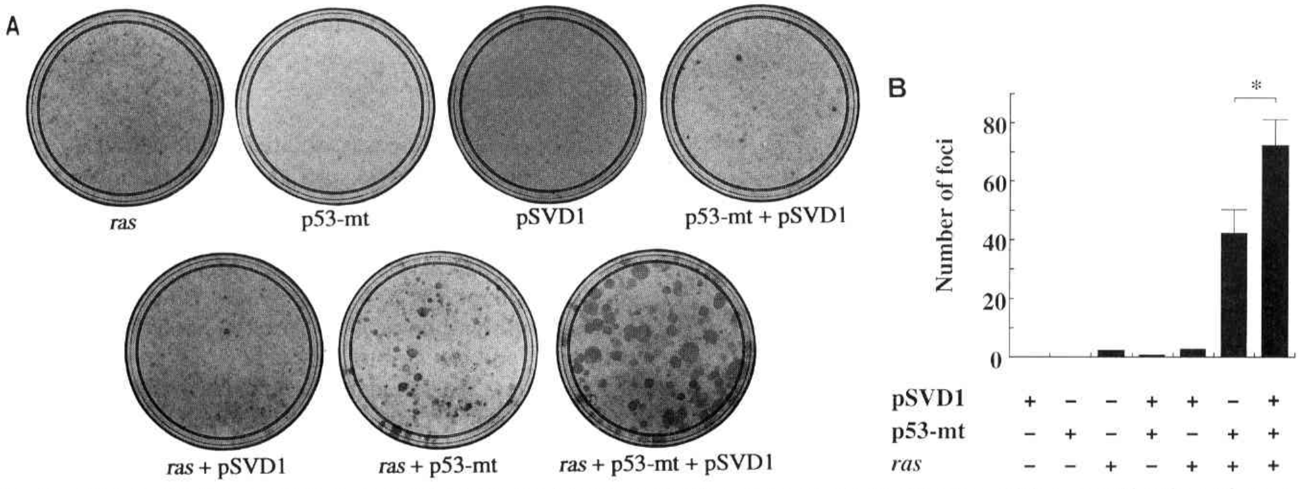

Fig. 2. The cyclin D1-expression plasmid augmented focus formation induced by transfection with a combination of *ras* and p53-mt. REFs for focus formation assay were incubated in D-MEM supplemented with 10% FBS. Transfections were performed with (+) and/or without (−) the indicated expression plasmid (5 μg each) as described under "Materials and Methods." Expression plasmids were: p53-mt, a mutated form of mouse p53; *ras*, activated allele of Ha-*ras*; pSVD1, human cyclin D1. A. A representative of 5 independent experiments. B. The number of foci found experimentally. Bar denotes SE. * $P<0.05$ by analysis of variance.

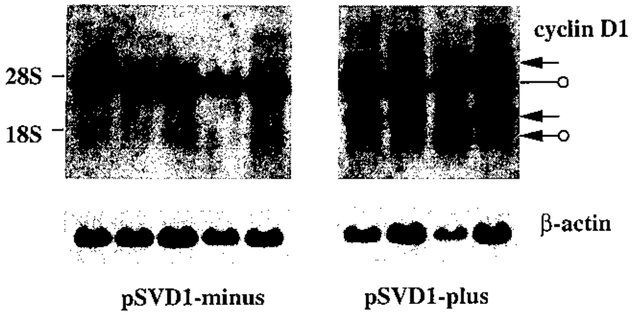

Fig. 3. Northern blot analysis for cyclin D1 expression in the cloned transformants. Total RNAs were extracted from the cloned transformants randomly chosen from foci generated after transfection with *ras* and p53-mt (pSVD1-minus) or with *ras*, p53-mt and pSVD1 (pSVD1-plus). Northern blot analysis was performed with the ^{32}P-labeled cyclin D1 probe as described under "Materials and Methods." Aberrant transcripts of cyclin D1 derived from pSVD1 are shown by arrows and endogenous ones by circles. The sizes of ribosomal RNAs are shown on the left. RNA loading of each lane was confirmed by rehybridization with a β-actin probe (lower part).

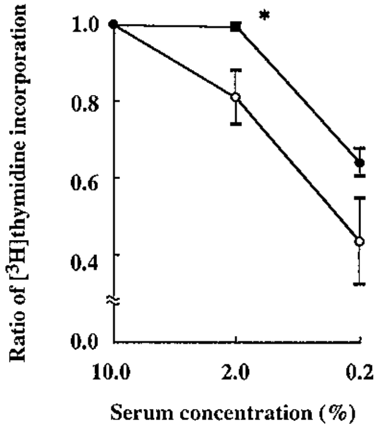

Fig. 4. Reduced serum dependency of the cloned transformants with the cyclin D1-expression plasmid. Transformants were cloned from foci generated by transfection with *ras* and p53-mt (n=5, ○) or with *ras*, p53-mt and pSVD1 (n=4, ●). [^{3}H]Thymidine incorporation for 24 h in D-MEM with the indicated concentration of FBS was determined in triplicate wells in each transformant. Data are presented as a ratio over that in the medium with 10% FBS. [^{3}H]Thymidine incorporation was assayed according to Danielpour *et al.*[21] Bars denote SE. * $P<0.05$ by Student's *t* test.

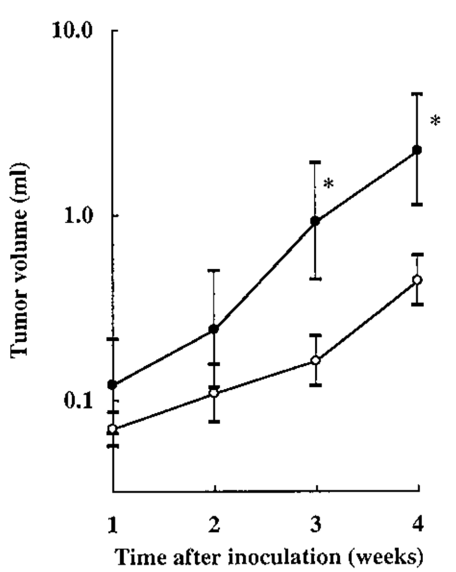

Fig. 5. *In vivo* growth of the cloned *ras*- and p53-mt-transformants with or without the cyclin D1 expression plasmid. The transformants were cloned from foci generated by transfection with *ras* and p53-mt (n=5, ○) or with *ras*, p53-mt and pSVD1 (n=4, ●). Each transformant (5×10^7 cells) was inoculated into nude mice twice. The volumes of resultant tumors were monitored weekly and were calculated as described under "Materials and Methods." The data are the average of two separate inoculations per transformant. Bar denotes SE. * $P<0.05$ by Student's *t* test.

transformants were inoculated twice into nude mice to observe *in vivo* growth. All the transformants easily generated aggressive tumors in nude mice. However, the pSVD1-plus transformants grew faster and afforded larger tumors than did the minus cells (Fig. 5). The difference in tumor volumes became statistically significant 3 weeks after inoculation.

DISCUSSION

The cyclin D1 gene was isolated as the PRAD1 (parathyroid adenomatosis 1) oncogene by positional cloning of tumor-specific clonal rearrangement breakpoints in parathyroid adenomas.[3-5] Cyclin D1 may also play a role in tumorigenesis in various other human tumors including malignant lymphoma with t(11;14)(q13;q32) (mantle cell lymphoma),[6, 7] breast cancer,[8, 9] esophageal cancer,[10] and squamous cell cancer of head and neck through gene rearrangement and gene amplification. Recently, several groups have attempted to establish the oncogenic potential of the cyclin D1 gene using rodent cell systems, and presented evidence that cyclin D1 could play an oncogenic role in certain situations *in vitro*.[14, 24, 25] In addition, Wang *et al.* reported that mammary hyperplasia and carcinoma developed in MMTV-cyclin D1 transgenic mice, and thus confirmed that cyclin D1 alone plays a role in carcinogenesis *in vivo*.[26] We and others[14] could not detect any significant focus formation with the cyclin D1 gene alone or in combination with *ras* or p53-mt in a rodent cell system, probably because of the different observation period and the differences of cell types and promoters used, compared to the other systems. However, the addition of a cyclin D1 expression plasmid to a combination of *ras* and p53-mt yielded an enhancement of focus formation, suggesting a collaborative effect of cyclin D1 in tumorigenesis, even over a period as short as 1 to 2 weeks, and with the weak SV40 promoter used in the current study.

The molecular mechanisms by which cyclin D1 contributes to tumorigenesis are not well defined. Quelle *et al.* reported that the forced expression of cyclin D1 shortened the G1 phase in rodent cells and thus shortened the doubling time, which would favor an oncogenic role of cyclin D1.[27)] In this study, the addition of pSVD1 did not change the doubling time. However, it did lead to lower serum dependency by the transformants, which was also noted by others.[27)] Deregulated expression of cyclin D1 may bypass at least a part of a growth factor-dependent signalling pathway and thus may contribute to tumorigenesis in collaboration with other genes such as *ras* and mutant p53.

Collaboration between *ras*, p53-mt and cyclin D1 in focus formation suggests a different role for each oncogene in tumorigenesis. Similar results were observed with *ras*, p53-mt and c-*myc*, where focus formation and metastatic potency were increased compared to that of any combination of two.[28)] In the current system, cyclin D1 may play a similar role to that of c-*myc*. While overexpression of c-*myc* usually has proliferative effects, forced expression of c-*myc* induces apoptotic death of fibroblasts in the absence of serum.[29)] Similarly, forced expression of cyclin D1 often compromises cell growth of normal or immortalized cells.[27, 30)] In the presence of *ras* and p53-mt, forced expression of cyclin D1 could stimulate cell growth. This suggests that cooperative oncogenic functions of other genes complement cyclin D1's role in tumorigenesis. Clinically, a significant association between loss of heterozygosity of the p53 locus and amplification of 11q13, where the cyclin D1 gene resides, was observed in breast cancers[31)] and may be relevant to our observation. Molecular mechanisms underlying the interaction of these three genes remain to be investigated.

Overexpression of cyclin D1 mRNA in transfected cells did not necessarily result in increased levels of its protein, probably due to posttranscriptional regulations.[27, 32)] Actually, overexpression of cyclin D1 mRNAs in t(11;14)(q13;q32)-bearing lymphoid tumors was not always associated with elevated cyclin D1 protein levels (T.M., unpublished observations). Even though we could not detect apparently increased levels of cyclin D1 protein, the introduced cyclin D1 expression significantly affected cell growth *in vitro* and *in vivo*. These observations suggest that deregulated expression of cyclin D1 could be important in tumorigenesis.

Several investigators reported that 11q13 amplification was associated with poor prognosis in different types of human cancers including esophageal[33)] and breast[34–36)] carcinomas. When inoculated into nude mice, the pSVD1-plus transformants grew faster and became larger than did the minus cells in the current study. Cells with an extra copy of the cyclin D1 gene appeared to have a growth advantage and could be selected through malignant progression, resulting in gene amplification and poor prognosis.

In sum, forced expression of the cyclin D1 gene causes the transformants to behave aggressively in the presence of *ras* and p53-mt, possibly due to decreased dependency upon growth factors or by higher growth potential *in vivo*. The current observations strengthen the significance of cyclin D1 overexpression observed in various human tumors and support the notion that deregulation of cyclin D1 expression in tumor cells represents a key step in multistep oncogenesis.

ACKNOWLEDGMENTS

We thank Drs. Akihisa Nagata and Hiroto Okayama for pcDneo plasmid, Dr. Thanos D. Halazonetis for pSV2humjun, Drs. Phillip W. Hinds and Steven Dowdy for p53-KH215 and pucEJ, and Dr. Masa-aki Matsuoka for COS7 cells. We are grateful to Dr. Kyoji Ikeda, University of Tokyo, for assistance in the assay for tumorigenicity. This work was supported in part by grants from the Ministry of Education, Science and Culture of Japan (to T. M.), an Araki Grant (to T. M.), NIH Grant CA 55909 (to A. A.) and an American Cancer Faculty Research Award (to A. A.).

(Received December 14, 1995/Accepted February 8, 1996)

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