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Research Article

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Autoantibodies to the Extracellular Domain of the Calcium Sensing Receptor in Patients with Acquired Hypoparathyroidism

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Abstract

Acquired hypoparathyroidism (AH) has been considered to result from an autoimmune process but the self-antigens have not been identified. We studied 25 patients with AH, of which 17 had type I autoimmune polyglandular syndrome and 8 had AH associated with autoimmune hypothyroidism. Five of 25 (20%) AH sera reacted to a membrane-associated antigen of 120–140 kD in human parathyroid gland extracts using immunoblot analysis. This is the exact size of the calcium sensing receptor (Ca-SR). The AH sera were then tested by immunoblot using a membrane fraction of HEK-293 cells transfected with Ca-SR cDNA. Eight of 25 (32%) AH sera reacted to a 120–140-kD protein, which closely matched that recognized by the anti-Ca-SR IgG raised in rabbits. The Ca-SR cDNA was translated in vitro into two parts in order to identify the antigenic epitopes. By using this technique, 14 of 25 (56%) AH sera were positive to the extracellular domain of the Ca-SR, whereas none of the AH patient sera reacted to the intracellular domain. The reactivity of the positive sera was completely removed after pre-absorption with the Ca-SR containing membranes. Sera from 50 patients with various other autoimmune diseases as well as 22 normal controls were also tested, and none of them was positive. In conclusion, the Ca-SR has been identified as an autoantigen in AH. (*J. Clin. Invest.* 1996; 97: 910–914.) Key words: autoantigen • epitope • parathyroid • autoimmune • polyglandular

Introduction

Acquired hypoparathyroidism (AH)¹ results from deficient parathyroid hormone (PTH) secretion without an identified

cause. This disease occurs as a common component of the type I autoimmune polyglandular syndrome (APS I) which presents in infants or young children. It may also appear as a sporadic disease in adults, most often in women affected by Hashimoto thyroiditis. APS I is characterized by mucocutaneous candidiasis, hypoparathyroidism and Addison disease, often accompanied by early onset pernicious anemia, chronic active hepatitis, alopecia and primary hypogonadism (1–4). APS I occurs either as an autosomal recessive disease, or as a seemingly sporadic disorder. Whereas APS I is not linked to genes within the HLA-DR region (5), the responsible gene has been recently mapped to chromosome 21q22.3 (6).

An autoimmune etiology for AH has been suggested because of its association with other autoimmune diseases (7), and by reports of autoantibodies directed against the parathyroid tissues in affected individuals. Autoantibodies to the parathyroid glands were first reported by Blizzard et al. (8). In that study, 38% of 74 patients with autoimmune hypoparathyroidism were found to be positive compared with only 6% of 245 healthy control subjects. The results from subsequent studies were controversial, since the antibodies often appeared to be directed against mitochondrial antigens, and mitochondrial rich cells are found within normal parathyroid tissues. Anti-mitochondrial antibodies are common in a variety of autoimmune disorders (9).

Over recent years, it has been discovered that in the case of organ specific autoimmune diseases, the self antigens targeted by autoimmune processes often involved specific receptors expressed on the surface of the targeted endocrine cells. Myasthenia gravis is such an autoimmune disease in which autoantibodies are produced against the acetylcholine receptor (10–12).

The parathyroid cell responds to falls in the concentration of the extracellular ionized calcium by elaboration of PTH, and the calcium-sensing receptor (Ca-SR) involved has been recently identified and its gene cloned (13). Autoantibodies from the sera of patients with sporadic adult onset hypoparathyroidism have been reported to bind to the cell surfaces of human parathyroid cells, resulting in an inhibition of PTH secretion (14). In addition, autoantibodies in the sera of patients with AH have been reported to be cytotoxic for cultured bovine parathyroid cells, by an antibody mediated cytotoxicity dependent on complement fixation and activation (15, 16). Whereas the above findings do constitute evidences for the presence of autoantibodies against parathyroid glands in AH, the nature of the targeted autoantigens has not been previously identified.

This study was to confirm the existence of parathyroid autoantibodies in AH, and to characterize the reacting human specific parathyroid autoantigens. We report here that parathyroid reactive autoantibodies are frequent in AH, and that the Ca-SR appear to be an important autoantigen target in the disease.

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1. Abbreviations used in this paper: AH, acquired hypoparathyroidism; APS, autoimmune polyglandular syndrome; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; Ca-SR, calcium sensing receptor; IDD, insulin-dependent diabetes; NBT, nitro blue tetrazolium.

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Methods

Patients. We examined sera from 25 patients with AH. Of these, 17 patients had APS I (all of them had AH, 14 had mucocutaneous candidiasis, 10 had Addison disease, and many had associated vitiligo, alopecia, chronic active hepatitis, and/or primary hypogonadism), 8 patients had adult-onset hypoparathyroidism associated with goiter and autoimmune hypothyroidism, confirmed by the presence of thyroid microsomal antibody and/or thyroglobulin antibody (Table I). We also studied sera from 10 patients with Addison disease, 10 with Graves disease, 12 with Hashimoto thyroiditis, 10 with insulin-dependent diabetes (IDD), and 8 with vitiligo (none of whom had AH), as well as 22 normal disease-free controls. No normal controls had any endocrine-associated serum autoantibodies, such as thyroid microsomal, thyroglobulin autoantibodies, or islet cell autoantibodies.

Antigen preparation. The human parathyroid glands were placed on ice in PBS with a mixture of protease inhibitors (1,10-phenanthroline, aprotinin, EDTA and benzamidine). The tissues were homogenized with a glass tissue grinder and centrifuged at 15,000 g to remove cell debris, nuclei and mitochondrial proteins, and used as antigen source in immunoblot.

Plasma membrane preparations from HEK-293 cells expressing the Ca-SR together with membrane preparations from wild type HEK-293 cells were kindly provided by Dr. Forrest Fuller (NPS Pharmaceutical, Salt Lake City, UT) and used as antigen sources in the immunoblot and absorption studies below. Dr. Fuller's group has shown that the Ca-SR protein is located to the surface of the transfected HEK-293 cells and perturbations in the calcium levels in the culture media elicit changes within the cells.

Immunoblot. The parathyroid gland extract and the HEK-293 cell membrane fractions were solubilized in SDS gel loading buffer containing DTT and heated for 3 min at 100°C before loading. After separation by a 8% SDS-PAGE, the proteins were transferred onto Immobilon-P membranes (Millipore Corp., Bedford, MA). The strips of the membrane were cut and incubated with 1% BSA in Tris-buffered saline and 0.05% Tween-20 (TBST) to block free potential binding sites. Test sera at 1/100 dilutions as well as purified IgG of a rabbit anti-Ca-SR antisera and IgG of pre-immune rabbit sera were incubated with the antigen-containing strips. The strips were then incubated with an anti-human or anti-rabbit polyvalent immunoglobulin alkaline phosphatase conjugate, and developed with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) (Promega, Madison, WI).

In vitro translation and immunoprecipitation. The human Ca-SR cDNA was obtained from Dr. Edward Brown (Brigham and Women's Hospital) and its extracellular and intracellular domains were amplified by PCR. The PCR products were positioned downstream of the SP6 promoter on the pcDNA3 construct. The recombinant plasmid was propagated in *E. coli* and purified by the Magic™ Minipreps System (Promega). The Ca-SR cDNA was transcribed and translated as described according to the manufacturer's instructions (Stratagene, La Jolla, CA). In brief, 1 µg circular plasmid DNA was transcribed in a 100 µl reaction for 2 h at 40°C, using SP6 RNA polymerase in the presence of RNasin. The translation was done using a methionine-free rabbit reticulocyte lysate (Promega) in a 50 µl reaction using 20% of the synthesized RNA as a substrate in the presence of 4 µl [³⁵S]methionine (10 mCi/ml) (Amersham, Arlington Heights, IL).

Once the translation reaction was complete, the translated prod-

ucts were examined by taking 5 µl aliquots mixed with 20 µl of SDS sample buffer. The samples were heated at 100°C for 3 min and subjected to a 10% SDS-PAGE. For autoradiography, the gels were dried and exposed to x-ray film (XAR-2 ready pack; Sigma Chemical Co., St. Louis, MO) overnight at room temperature. Since Ca-SR is a glycoprotein, canine pancreatic microsomes (Promega) were added to the translation reaction mixture in order to obtain the mature glycosylated receptor.

For characterization of the autoantibody reactivities, the translated products (50,000 cpm) were incubated at 4°C overnight with 2 µl of sera diluted in PBS with a final volume of 100 µl. The immunocomplexes were washed three times with ice-cold PBS and incubated with protein A-Sepharose beads for another 45 min. After washing, 50 µl of the SDS gel loading buffer were added to the bead and boiled for 3 min. Autoradiography were performed as above.

Absorption of autoantibodies with recombinant Ca-SR. The patient sera (2 µl) were incubated for 2 h at room temperature with 1 mg recombinant Ca-SR expressed by HEK-293 cell diluted in 50 µl PBS. This mixture was then centrifuged (13,000 g, 15 min), and the supernatant was again subjected to immunoprecipitation with the in vitro translated extracellular domain of the Ca-SR as an antigen source, to learn whether the positive band (see later) had been removed.

Results

Autoantibodies were detected against a parathyroid specific protein in 5 of 25 (20%) of the AH sera by immunoblot. Two patients had APS I and 3 had adult onset AH. The autoantibodies reacted with a doublet 120–140 kD protein in the parathyroid gland extract (Fig. 1). Sera from 50 patients with other autoimmune diseases as well as 22 normal controls were also tested, and none of them was positive.

Since the parathyroid 120–140 antigen has the same molecular weight as the Ca-SR dependent upon its degree of glycosylation, we tested the possibility that the receptor itself was the autoantigen by three different experimental approaches.

In the first approach, the AH sera were tested by immunoblot using a membrane fraction of HEK-293 cells transfected with Ca-SR cDNA. The patient sera reacted to a 120–140-kD protein (Fig. 2, lane 2), which closely matched that recognized by the anti-Ca-SR IgG raised in rabbit (Fig. 2, lane 3). Eight of 25 AH patient sera (32%, 3 APS I and 5 adult onset AH) including the above-mentioned five positive sera reacted to the Ca-SR from this source, but none of the control sera did so. In addition, the eight positive AH patient sera did not react to nontransfected or wild type HEK-293 cells which did not express Ca-SR proteins (Table II).

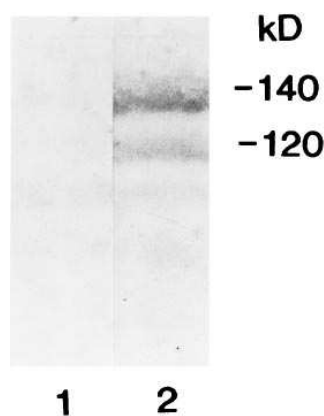


Figure 1. Immunoblot analysis with human parathyroid gland extract. The parathyroid gland extract was solubilized and separated by a 8% SDS-PAGE. After separation, the proteins were transferred onto Immobilon-P membranes. Immobilon-P strips containing the parathyroid extract were incubated with normal sera (lane 1) and AH sera (lane 2). The reactivities of the autoantibodies were visualized by an alkaline phosphatase mediated BCIP/NBT system.

Table I. Characteristics of AH Patients

Subject	Number	Gender	Age of onset (range)
AH in APS I	17	10F and 7M	1–12 yr
AH in adult	8	8F and 0M	31–53 yr

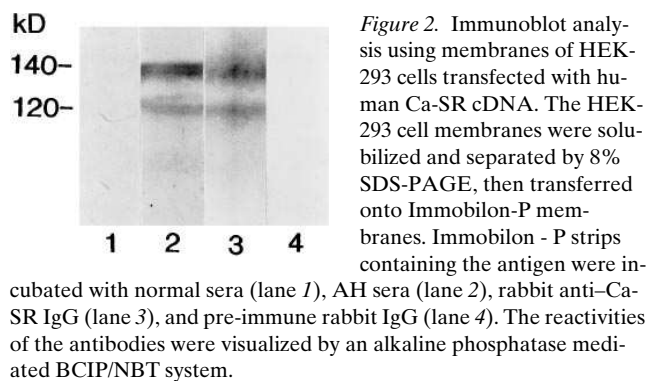


Table II. Autoantibody Reactivity to Recombinant Ca^{2+} -sensing Receptor (Immunoblot)

Ag source	AH patients	Normal	Rabbit anti-Ca-SR IgG	Pre-immune rabbit IgG
Transfected HEK-293 cell	8/25 (32%)	0/22	+	—
Wild type HEK-293 cell	0/25	0/15	—	—

In the second approach, the Ca-SR was translated *in vitro* into two parts in order to identify the antigenic epitopes reactive to the putative autoantibody that we had discovered. Overlapping extracellular (residues 1–613) and intracellular (residues 580–1085) domains of the Ca-SR were expressed as shown in Fig. 3. The extracellular domain was translated as shown in Fig. 4. Two bands with molecular weights of 46 and 60 kD are seen in lane 1. Glycosylation occurs with the addition of canine pancreatic microsome membranes. As can be seen in lanes 2–5, this step induced one additional band to appear at 70 kD, meanwhile, the intensity of the 60-kD band decreased by 50% as expected for glycosylated proteins. That is

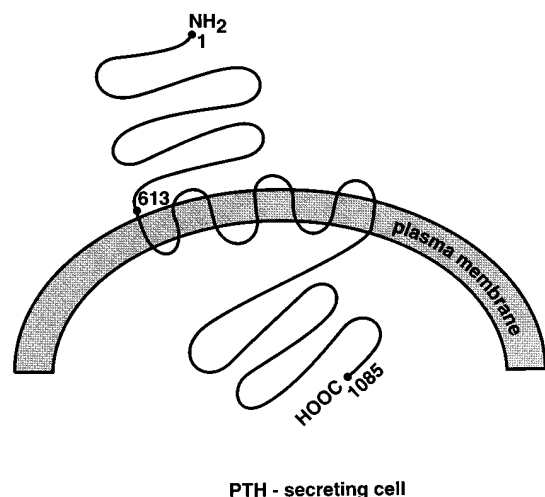
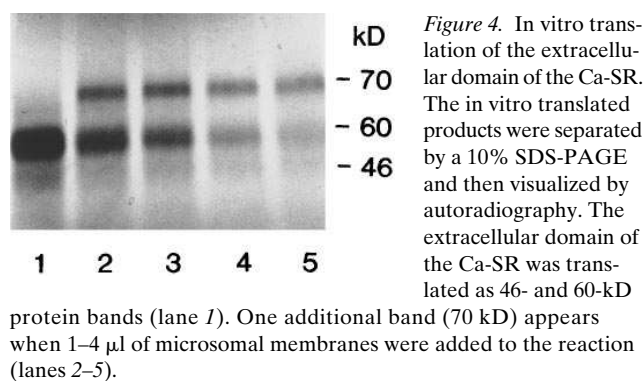


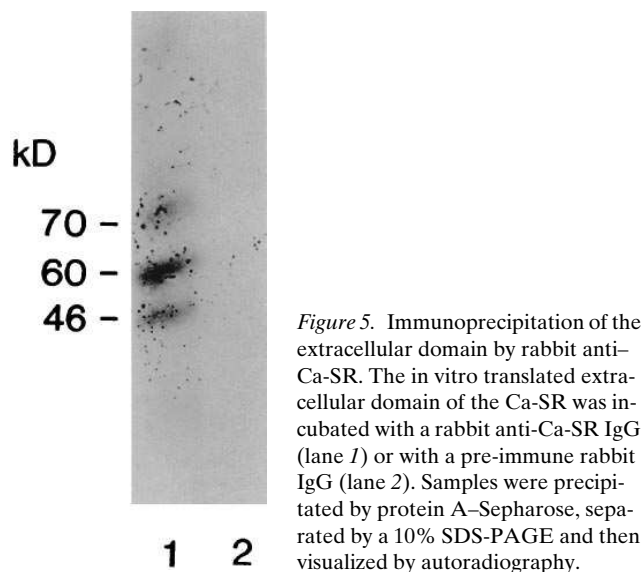
Figure 3. Diagram of the strategy for *in vitro* translation of Ca-SR. The full length Ca-SR cDNA encode 1085 amino acid. The extracellular domain (1–613) and intracellular domain (580–1085) were translated separately by a rabbit reticulocyte system.



to say, the 60 kD is the non-glycosylated form and 70 kD is the glycosylated form. The 46-kD band, however was unexpected. To find out whether it belong to the Ca-SR or was due to the background of the system, we immunoprecipitated the translated extracellular domain by the rabbit anti-Ca-SR antibody. This rabbit antibody was raised against a peptide of the extracellular domain. As shown in Fig. 5, the antibody recognized all three bands, suggesting that the 46 kD band is also a portion of the Ca-SR, perhaps a degraded or truncated product.

The patient sera reacted to 60- and 70-kD forms of the extracellular domain but not the 46-kD band (Fig. 6) indicating that the autoantibodies recognized different epitopes compared to the rabbit antibody. By using this technique, 14 of 25 (56%) AH sera were positive. Six had APS I and 8 had adult onset AH. Furthermore, glycosylation is not required in the formation of all autoantibody reactive antigenic epitopes since both the nonglycosylated (60 kD) and glycosylated (70 kD) proteins were so recognized. None of the control sera reacted to the extracellular domain of the Ca-SR.

In the third approach, the positive sera were pre-incubated with the HEK-293 membranes containing the Ca-SR. The reactivity of the sera was completely removed after the pre-absorption. As shown in Fig. 7, the AH sera reacted with a 60-, and 70-kD protein before absorption (lane 1) and the reactivity disappeared after the absorption (lane 2).



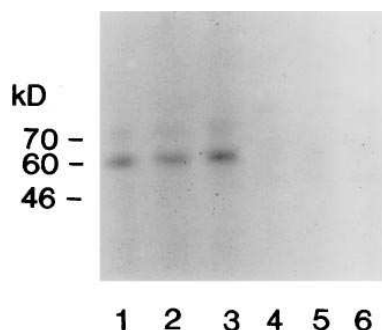


Figure 6. Immunoprecipitation of the extracellular domain of the Ca-SR by AH sera. The *in vitro* translated extracellular domain of the Ca-SR was incubated with AH sera (lanes 1–3) or with normal control sera (lanes 4–6). Samples were precipitated by protein A–Sepharose, separated by a 10% SDS-PAGE and then visualized by autoradiography.

The cytosolic domain was translated as a 60-kD protein and no glycosylation occurred after exposure to the microsomal membranes as expected. None of the patient sera reacted with the cytosolic or intracellular domain of the Ca-SR.

In summary, 14 (56%) of AH patient sera reacted to the extracellular domain of the recombinantly expressed Ca-SR, whereas none of the 25 AH patient sera reacted to the intracellular domain of the molecule. The 14 antibody positive patients which responded to the extracellular domain of the Ca-SR included all 8 positive patients that had reacted to the transfected HEK-293 cells. The autoantibody frequencies might have been higher if newly diagnosed patients had been exclusively studied. None of the 22 normal control sera reacted to either domain of the Ca-SR. Sera from 50 patients with other autoimmune diseases were also tested, and none of them reacted to either domain of the Ca-SR (Table III). The Ca-SR autoantibodies tend to disappear long after the onset of the disease. In fact, among the 25 AH patients we studied, 7 of them have AH for more than 5 years and only one of the 7 patients was positive for Ca-SR autoantibody (14%) compared to the 18 patients who had AH for less than 5 years, of which 13 were antibody positive (72%) when studied.

Some of our most positive sera were sent to Dr. Fuller's lab. They incubated the sera with live transfected HEK-293 cells and measured the change of intracellular Ca^{2+} level, but could not demonstrate an effect.

In this study, the sex incidence of antibody negative patients is 28% (5/18) for female and 86% (6/7) for male. Therefore, gender happened to be related to the lack of autoanti-

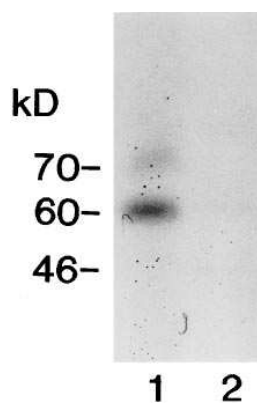


Figure 7. Absorption studies with HEK-293 cells. The extracellular domain of the Ca-SR was immunoprecipitated by a positive AH sera before (lane 1) and after (lane 2) pre-absorption with HEK-293 cell membranes containing the Ca-SR. Samples were precipitated by protein A–Sepharose, separated by a 10% SDS-PAGE and then visualized by autoradiography.

Table III. Autoantibody Reactivity to *In Vitro* Translated Domains of Calcium Sensing Receptor (Immunoprecipitation)

Subject	Antigen Source	
	Ca-SR extracellular	Ca-SR intracellular
AH	14/25 (56%)	0/25
Addison disease	0/10	0/10
Graves disease	0/10	0/10
Hashimoto thyroiditis	0/12	0/12
IDD	0/10	0/10
Vitiligo	0/8	0/8
Normal control	0/22	0/22

bodies to the Ca-SR and the antibodies are less detectable in male patients.

Discussion

Our studies confirmed the autoimmune nature of AH, and document the presence of autoantibodies in patients with AH which target the Ca-SR. The autoantigen is disease specific since it was only recognized by the sera from patients with AH and not from those with other autoimmune diseases. The antigenic epitope is exclusively localized to the extracellular domain of the Ca-SR.

Most of the Ca-SR autoantibody positive patients (including five AH in the context of APS I and all eight adult-onset AH in association with hypothyroidism) were females. This finding is consistent with findings in other autoantibody mediated disease targeted at membrane receptors which occur predominantly in females. Four of our adult-onset AH patients developed their disease and had the Ca-SR autoantibodies detected after they had delivered babies, another two adult-onset AH patients developed their disease after menopause, while one who presented with AH in the context of APS I began her disease at the onset of her menses. These findings suggest a possible influence of female hormones in the manifestation of the disease.

That autoantibodies to Ca-SR were absent from some AH in the context of APS I could be explained by the complete loss of the autoantigen needed to drive their formation, before we could study them. In fact, two of the Ca-SR autoantibody negative AH patients had developed their disease 32 years previously, while another two autoantibody negative AH patients had their diseases for more than 10 years at the time of this study. However, we were able to collect a serum sample immediately after the onset of AH from a 34 yr-old-female who developed AH after an infection by influenza and she happened to be very positive for Ca-SR antibody. A general characteristic of all autoimmune diseases is that there are remissions and exacerbations of the underlying pathogenic processes involved over time. With IDD, islet cell autoantibodies (ICA) disappear following clinical onset of disease when the pancreatic β cells are destroyed, and the ICA reactive self antigens have disappeared (17). In some diabetic patients, even with the combination of different well defined antigens, autoantibodies just simply are never detected. AH may have a course like this in respect to the Ca-SR autoantibody also. Alternatively, different antigens may exist in different patients or

the possibility that in some patients they simply do not appear at any time. We are planning to explore other potential autoantigens in a separate study by our research group.

The Ca-SR is over expressed on HEK-293 cell membranes and contains much more Ca-SR than the native parathyroid extracts. This may explain why more of the sera were positive when the HEK-293 cell membranes were used as antigen source. The in vitro translated extracellular domain of Ca-SR had much less background than the transfected HEK-293 cells and this may explain why more of the AH patients again were found to be positive when in vitro translated extracellular domain of Ca-SR was used as the antigen source in immunoprecipitation studies.

The Ca-SR appeared as a 120–140-kD band on the immunoblot of the native human parathyroid protein due to differential glycosylation of the receptor components. However, this differential glycosylation did not appear to affect the antigenic structure of the Ca-SR since the 120–140 kD was recognized by both rabbit antibody and the AH patient sera.

The role of Ca-SR autoantibodies in the pathogenesis of AH is not known. The pathogenic event might also involve cytotoxic lymphocytes rather than autoantibodies. The specificity of our results, however, argue best for a possible role of autoimmunity to the Ca-SR in AH. Our finding of autoantibodies to the Ca-SR in AH could lead to the development of a diagnostic test for the disease, as well as possibly provide antigen mediated immunotherapies based upon the use of recombinant protein antigen as a therapeutic agent to restore immune tolerance in AH.

Acknowledgments

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