# MACROMOLECULAR STRUCTURE OF BASEMENT MEMBRANE COLLAGENS

## Identification of 7 S collagen as a crosslinking domain of type IV collagen

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### 1. Introduction

Type IV collagen is known as a major component of basement membranes [1]. It contains two different polypeptide chains (review [2]) which differ from the α chains of the interstitial collagens type I, II and III in amino acid composition [1], a larger $M_r$-value [3–7] and in frequent interruptions of the triple helix by non-helical regions [8]. These interruptions are responsible for the high sensitivity of basement membrane collagen to proteolytic attack. In [9,10] unusual collagen structures were isolated from basement membranes of a tumor matrix, placenta, lens capsule and kidney after limited proteolytic digestion. These structures named 7 S collagens appeared in a long and a short form, with app. $M_r$ 360 000 and 200 000, respectively. The data suggested that 7 S collagen is composed of several triple helical segments with a high denaturation temperature of 70°C, due to multiple disulfide bonds between the chains [10]. Because of the high stability of the triple helix, 7 S collagen is resistant to intensive treatment with pepsin or bacterial collagenase under conditions where other triple helical structures would be digested [9].

In previous studies it could not be decided whether 7 S collagen represent a special domain of type IV collagen involved in intermolecular crosslinking or a fragment of a new, unusual type of collagen. By using the rotary shadowing technique we have now compared the size and dimensions of 7 S collagen and of polymeric forms of type IV collagen from a mouse tumor and human placenta. The data demonstrated that 7 S collagen is a domain of type IV collagen in which 4 triple helical molecules are held together by disulfide bridges and presumably by other covalent crosslinks.

### 2. Experimental

Collagenous proteins dissolved by treatment of a mouse tumor matrix with trypsin (Worthington) for 4 h at 20°C and pH 7.9 [10] or of human placenta with pepsin (Boehringer, Mannheim) for 20 h at 4°C in 0.5 M formic acid [11]. The long form of 7 S collagen was prepared by digesting the dissolved material with bacterial collagenase (CLSPA, Worthington) for 24 h at 20°C and pH 7.4 or with pepsin in 0.5 M acetic acid at 20°C for 72 h and was chromatographically purified. The short form of 7 S collagen was produced by a second collagenase treatment of the long form with bacterial collagenase at 37°C [10]. Samples of 7 S collagen were completely reduced under non-denaturating conditions followed by alkylation with ethylene imine and dialysis against 0.1 M acetic acid in order to dissociate the molecule [10].

Polymeric type IV collagen was dissolved by treatment of human placenta suspended in 0.5 M formic acid with pepsin at 4°C for 6 h. After precipitation of the dissolved collagenous proteins with 6% NaCl, type IV collagen was then separated from type I and V collagens by precipitation with 1.8 M NaCl at pH 7.4 [11]. Polymeric type IV collagen from a mouse tumor matrix was solubilized in a similar way and purified by salt precipitation and chromatography on DEAE-cellulose [12].

For electronmicroscopic investigations the rotary shadowing technique was adapted from [13]. Protein

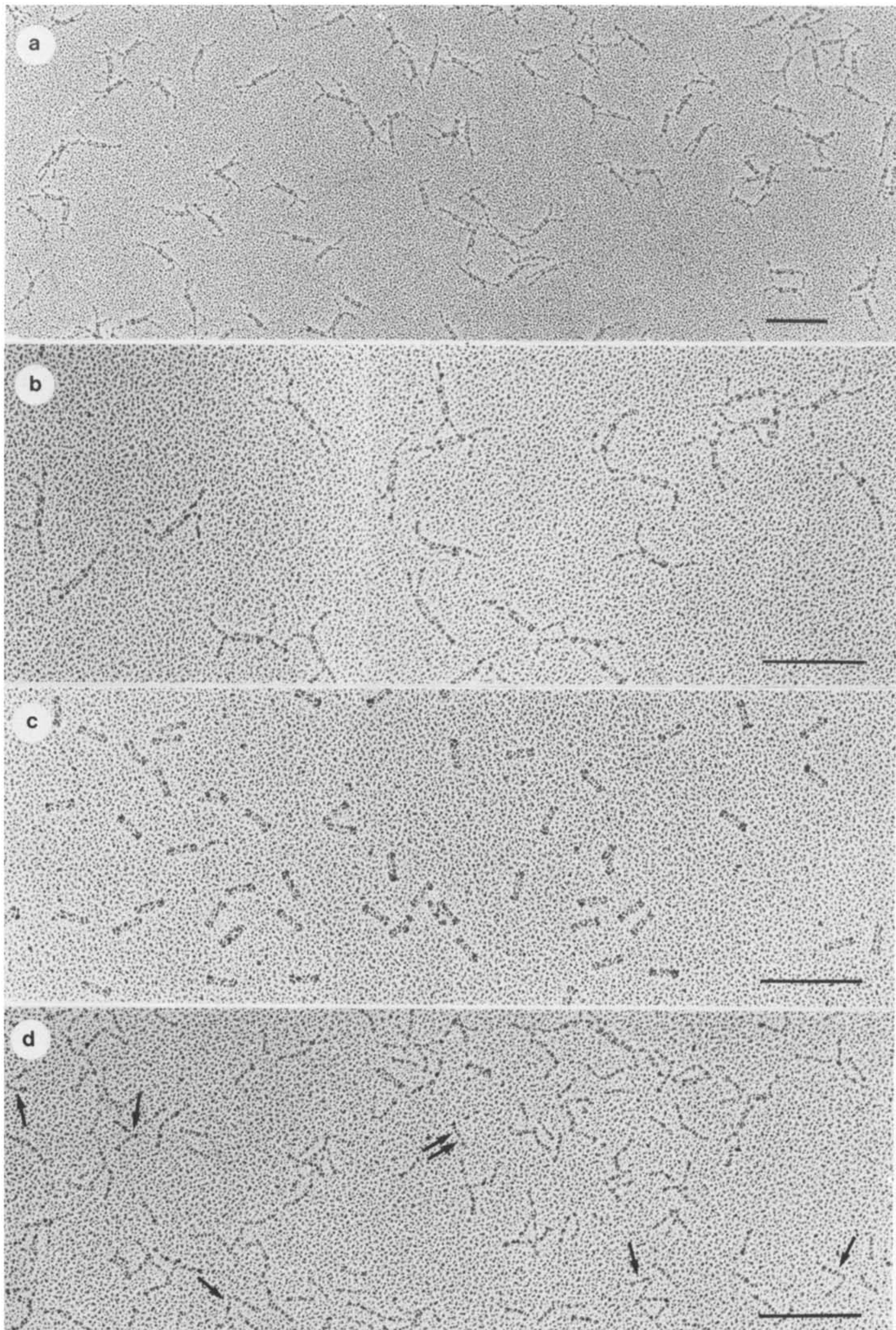


Fig.1. Electron micrographs of various forms of 7 S collagen: (a) long form from human placenta after collagenase digestion at 20°C; (b) long form from human placenta after pepsin digestion at 20°C; (c) short form from mouse tumor after collagenase digestion at 37°C – note the loss of the 4 arms present in the long form; (d) short form as in (c) but under non-denaturing conditions and exposed to 0.1 M acetic acid. The central domain of 7 S collagen dissociated into 2 parts of similar length but smaller diameter (↑↑). V-shaped structures are the 2 parts are still held together at one end (↑). Bars indicate the length of 100 nm.

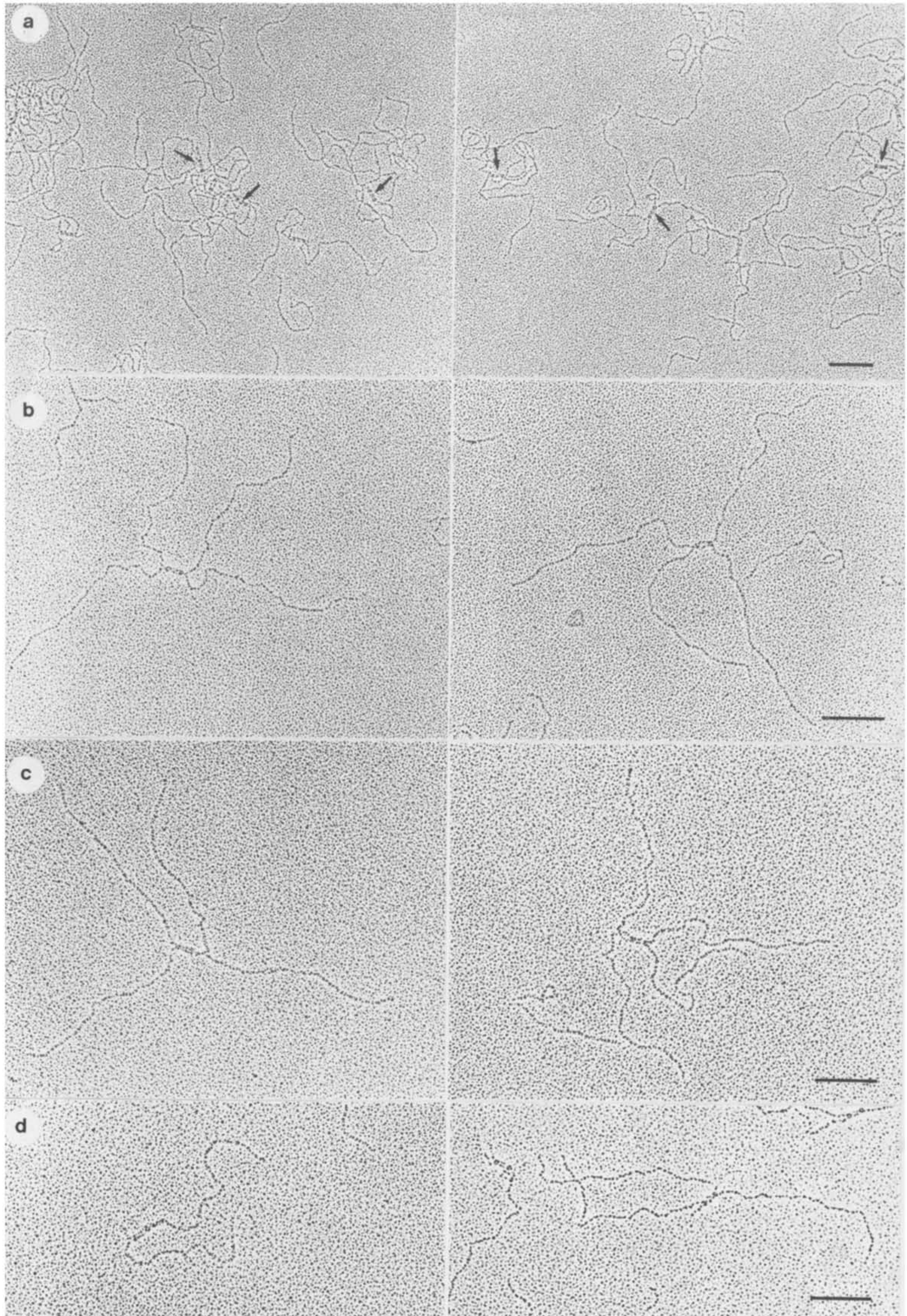


Fig.2. Electron micrographs of polymeric type IV collagen obtained by limited pepsin digestion from mouse tumor (a,b) and human placenta (c,d): (a) general view; (b,c) higher magnification; (d) polymeric intermediates where 1 or 2 of the long arms have been split off. Arrows indicate the central domain of 7 S collagen. Bars indicate the length of 100 nm.

samples were dissolved in 0.05 M acetic acid (20–30 μg/ml) and after addition of an equal volume of glycerol sprayed onto freshly cleaved mica discs from a distance of 30 cm. The samples were then immediately brought into a vacuum chamber of an Edwards vacuum coater model 306 and evacuated to $1 \times 10^{-5}$ torr. An Edward's electron-bombarded source was used for shadowing the proteins with platinum at an angle of 9°, followed by carbon coating at 90°. Platinum wire (length 5 cm, diam. 0.2 mm) was coiled around a 2 mm diam. tungstic rod and completely evaporated at 40 kV and an emission current of 50 mA. The distance to the mica discs mounted on a rotating table (120 rev./min) was 15 cm. Carbon was evaporated at 40 kV and an emission current at 50–100 mA for 10 s. The replicas were cut into 2–3 mm diam. fragments, floated onto distilled water and picked up on 400 mesh copper grids. Specimens were examined in a Siemens electron microscope, Elmiscope 102 at 100 kV using a 50 μm objective aperture.

Electron micrographs were taken at magnifications of $2–5 \times 10^4$. The magnification was calculated by photography under the same electron optical conditions of T4 phage tails (length 950 nm) negatively stained with uranyl acetate and catalase crystals (periodicity 8.75 nm). Type I collagen was used as a further control and showed a length of 297 nm. The absolute error in the magnification factor is estimated to be ±5%. The length of the arms of polymeric type IV collagen was measured on photographs at a total magnification of $12–15 \times 10^4$ using a Nunomic electronic graphic calculator. The dimensions of 7 S collagen were measured on photographs at a total magnification of $15 \times 10^4$ and $25 \times 10^4$ using a glass scale (graduation 0.1 mm) under a stereomicroscope.

## 3. Results

Rotary shadowing visualized 7 S collagen as rod-like, branched structures (fig.1) and polymeric type IV collagen mainly as flexible, long strands (fig.2) with a remarkably low variance in the length of the different segments (table 1). The central region, common to both forms of 7 S collagen, was a rod-like, 30 nm long particle (fig.1c). It exhibited at both ends a thickening with diam. 7–8 nm, connected by a thinner central part which frequently appeared to be divided into 2 strands. Additional structures were found in the long form of 7 S collagen and consisted of 4 apparently identical arms (length 28 nm) which extended from the central rod in a symmetric fashion. Identical pictures were observed for 7 S collagens prepared by either collagenase (fig.1a) or pepsin (fig.1b) digestion. Reduction of disulfide bridges in the central core split the short form of 7 S collagen along its axis into thinner, 27 nm long strands (fig.1d). It was often observed that 2 of these rods were still connected at one end forming a V-shaped structure. Reduction of the long form of 7 S collagen produced a similar dissociation into thinner strands (not shown).

Polymeric forms of type IV collagen, solubilized by limited pepsin digestion at 4°C were found to consist of abundant amounts of spider-like structures

Table 1
Dimensions of 7 S collagen (short and long form) and polymeric type IV collagen observed after rotary shadowing in the electron microscope

| Preparations | Origin | Structures evaluated | Length (nm ± SD) | No. molecules measured |
|---|---|---|---|---|
| 7 S collagen (short form) | Mouse | Central part | | |
| | | – unreduced | 30.4 ± 0.5 | (78) |
| | | – reduced | 27.0 ± 0.6 | (104) |
| 7 S collagen (long form) | Human | Central part | 30.9 ± 1.0 | (87) |
| | | Arms | 27.8 ± 1.3 | (110) |
| Type IV collagen (polymers) | Mouse | Central part | 30.7 ± 0.9 | (31) |
| | Human | Central part | 30.5 ± 1.2 | (23) |
| | Mouse | Arms | 358 ± 9.4 | (58) |
| | Human | Arms | 356 ± 10.8 | (63) |

(fig.2a,b). The major features of this structure were four, 357 nm long threads connected at one end by a domain with the morphologic appearance of the central region of 7 S collagen. Less frequently structures were observed which had lost 1–3 long arms but had retained the short stretches of thin strands characteristic for the long form of 7 S collagen (fig.2d). Further pepsin treatment resulted in a cleavage of the 4-stranded structures 28 nm away from the crosslinking domain, leading to the long form of 7 S collagen and single-stranded fragments varying in length from 140–327 nm. The proportion of smaller fragments increased with intensity of pepsin treatment (not shown).

While the length of the structures could be determined with high precision, the diameters could not be accurately measured because of the thickness of the platinum layer surrounding the molecules. Single-stranded structures appeared after rotary shadowing 2.5–3 nm thick and 4-stranded structures at the core of 7 S collagen showed diam. 6–8 nm. As discussed below, chemical and physical data indicated that these structures represent single triple helices (diam. 1.4 nm) or the association of 4 triple helices (diam. 4 nm). A similar discrepancy has been observed in analyzing other proteins by rotary shadowing [14,15].

## 4. Discussion

The polymeric type IV collagen appeared as 4 strands, each 387 nm long, which very likely represent 4 triple helical molecules. They are connected via their 30 nm long terminal regions forming the central 7 S domain. Based on a residue length of 0.286 nm measured parallel to the axis of the triple helix [16], the type IV collagen chains are 1350 amino acid residues long. Using an average residue $M_r$ of 110, including the carbohydrate content of type IV collagen, one chain is calculated to be $M_r$ 150 000 and the entire polymeric type IV collagen consisting of 4 triple helical molecules $\sim 2 \times 10^6$ $M_r$. This explains why such material does not enter polyacrylamide gels in electrophoresis [11]. The fragmentation of the polymers by proteolysis starts with a scission of the peptide chains at a region 28 nm away from the central 7 S domain. In this area intact polymers often show a sharp kink, indicating a more flexible non-triple helical structure, sensitive to proteolytic attack. Similar, non-triple helical segments may exist along the molecule [8] explaining the continuous production of shorter strands upon pepsin treatment. Several of these shorter strands have been characterized in chemical studies [3–8,11,12].

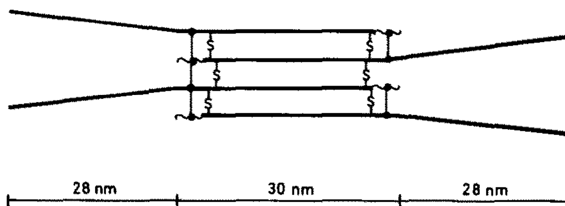


Fig.3. Model of 7 S collagen (long form) representing the terminal crosslinking region of basement membrane collagen type IV. Each thick line represents a triple helical segment, the thin lines indicate non-helical sequences at the end of the helix. The molecules are connected by disulfide bridges (–S–) and by non-reducible crosslinks (•——•) presumably derived from oxidized lysine residues. The number and positions of crosslinks are tentative. The 4 triple helices are presumably arranged in a tetragonal array.

The electron microscopic finding that the central 7 S domain is formed by the end regions of 4 type IV collagen molecules (fig.3) is compatible with diam. 3.1–3.4 nm calculated for 7 S collagen from its hydrodynamic properties [10]. According to the dimensions of 7 S collagen, the peptides forming the short and long form are 105 and 203 residues long. Using the amino acid composition and the carbohydrate content the chains are calculated to be $M_r$ 14 000 and 26 000, respectively. About 20% higher $M_r$ values were pre-determined by ultra-centrifugation of 7 S collagen consisting of 12 of these chains [9,10]. The differences may be attributed to some non-helical sequences at the very ends of the peptide chains in 7 S collagen [10] which may be folded in a more globular shape (thickening in the end regions of the central domain).

In the central domain of 7 S collagen the 4 type IV molecules are crosslinked by disulfide bridges [9,10] and presumably by lysine-derived crosslinks [17]. Some of the peptide chains produced from completely reduced 7 S collagen showed a $M_r$-value similar to that calculated above from the length of the molecules. Most of the other peptides corresponded to multiples of these $M_r$-values but were similar in composition and antigenicity [9,10]. This suggested that the peptides are oligomers of the smallest peptide units which are connected by non-reducible bonds. The identification of 7 S collagen as a major crosslinking domain (fig.2) and the detection of frag-

ments in reduced 7 S collagen twice as long as the original strands (V shaped structures in fig.1d) support this interpretation.

Intermolecular crosslinks in interstitial collagens are formed by a lysine residue located in the non-helical sequence at both ends of the $\alpha$ chains. Due to the D (= 67 nm) staggered array of the molecules in the fibrils it interacts with a hydroxylysine residue located 27 nm away from the end of another molecule [18]. This distance is remarkably similar to the length of the central domain of 7 S collagen, suggesting some homology between the crosslinking sites of interstitial and basement membrane collagens. We propose a model of 7 S collagen (fig.3) where the triple helices are aligned in an anti-parallel fashion. Non-reducible lysine derived crosslinks may be formed between non-helical sequences located at one end of the chains and adjacent helical structures of anti-parallel molecules. Intermolecular disulfide bridges may be arranged either in a symmetric or asymmetric manner.

The symmetric structure of polymeric type IV collagen strongly suggests an unique arrangement of the molecules in the basement membrane. The most likely model would be a regular but loose mesh work of single molecules, which implies two crosslinking sites, one of which has been removed during limited proteolysis. Preliminary studies on the acid extracted form of type IV collagen [4] have supported this possibility (unpublished). This type of supramolecular organization is quite different to the fibrillar assembly of interstitial collagens [18], in which staggered molecules interact in a lateral fashion over their entire length. Our model of a type IV collagen meshwork agrees with X-ray diffraction studies on stretched lens capsules [19] indicating a lack of fibrillar structures in basement membranes.

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