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Analysis of the Ternary Interaction of the Red Cell Membrane Skeletal Proteins Spectrin, Actin, and 4.1†

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ABSTRACT: Spectrin dimers interact weakly with F-actin under physiological solvent conditions (with an association constant of about 5×10^3 M^{-1} at 20 °C). In the presence of the membrane skeletal constituent, protein 4.1, strong binding is observed; an analysis of the profiles for formation of a ternary complex leads to an association constant of about 1×10^{12} M^{-2}. This association becomes weaker at low ionic strength, whereas the opposite applies to the spectrin–actin interaction. The stability of the ternary complex is maximal at physiological ionic strength and somewhat above. The effect of temperature in the range 0–20 °C on the formation of the ternary complex is small, whereas the spectrin–actin interaction almost vanishes at low temperature. There is no detectable calcium sensitivity in either the binary or the ternary system within the limits of precision of our assay. The ternary complex resembles the natural system in the membrane in that the actin is resistant to dissociation and unavailable in the deoxyribonuclease assay; after selective proteolytic destruction of spectrin and 4.1, all the actin becomes available. In the absence of 4.1, spectrin dimers do not measurably protect the actin against dissociation.

The shape and mechanical properties of the mammalian red blood cell are determined by a network of proteins on the cytoplasmic surface of the membrane. The constituents of this complex are spectrin, actin, and proteins 4.1 and 4.9, the latter being a minor component. For the formation of a highly stable continuous network under physiological salt conditions it was shown (Ungewickell et al., 1979) that the actin must be polymerized, the spectrin must be tetrameric, and 4.1 must be present. In the absence of 4.1, interaction of spectrin with F-actin is still observed (Brenner & Korn, 1979) but is clearly very much weaker (Cohen & Foley, 1980). The spectrin occurs in the cell predominantly as a tetramer (Ungewickell & Gratzer, 1978; Liu & Palek, 1980), made up of two heterodimers associated head-to-head. The 4.1 binding sites are near the distal ends of the tetramer (Tyler et al., 1979), and the tetramer is thus divalent with respect to 4.1 and actin (Ungewickell et al., 1979). The binding of 4.1 to spectrin has been studied (Tyler et al., 1980; Wolfe et al., 1982), but that of red cell 4.1 to actin, which by implication should occur, appears not yet to have been detected under physiological solvent conditions and must thus be supposedly weak in the absence of spectrin.

Red cell spectrin is the archetypal member of a class of elongated proteins, all of which bind to F-actin (Levine & Willard, 1981; Goodman et al., 1981; Davis & Bennett, 1982; Repasky et al., 1982; Glenney et al., 1982); protein 4.1 has similarly been found in various cell types (Cohen et al., 1982; Spiegel et al., 1982). The binding of brain spectrin (fodrin) to actin is also promoted by 4.1 (Burns et al., 1983). We have studied the nature of the interaction between actin, red cell spectrin, and 4.1 and the thermodynamic characteristics of what appears still to be the only known ternary protein system of this kind to have been characterized.

Materials and Methods

Protein Preparation. Proteins were prepared from human red cells that had been stored for less than 1 week. Spectrin

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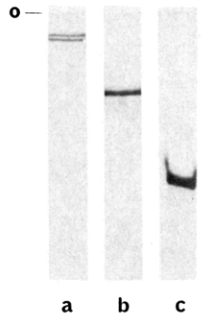

FIGURE 1: Gel electrophoresis in the presence of SDS of purified protein preparations used in this study: (a) spectrin, (b) 4.1, and (c) muscle actin.

dimers and tetramers were prepared by extraction of ghosts at low ionic strength at 35 and 4 °C, respectively, and purified by chromatography on Sepharose 4B, as described elsewhere (Ungewickell & Gratzer, 1978). F-Actin was extracted from rabbit or chicken breast muscle and purified by the method of Spudich & Watt (1971). Protein 4.1 was prepared either by dissociation of isolated cytoskeletons (Sheetz, 1979) in 1 M tris(hydroxymethyl)aminomethane (Tris) (Burns et al., 1983) followed by chromatography on Sepharose 6B or by extraction from membranes with Tween 20 followed by ion-exchange chromatography (Becker et al., 1983). Preparations were screened for purity by gel electrophoresis in the presence of sodium dodecyl sulfate (Laemmli, 1970), as shown in Figure 1. The activity of 4.1 prepared by the second procedure was tested by affinity chromatography on matrix-immobilized spectrin (Wolfe et al., 1982). Fresh preparations were in general about 80% active, as judged by the fraction of the protein retained on a column of the matrix. Spectrin solutions were concentrated by precipitation with an equal volume of cold saturated ammonium sulfate and 4.1 by dialysis against polyethylene glycol (Aquacide), followed by dialysis against the appropriate buffer.

Binding Assays. Binding of spectrin dimer to F-actin was determined by pelleting in the ultracentrifuge. The spectrin dimer was radioiodinated with the reagent of Bolton & Hunter (1973). In a typical procedure, F-actin, generally at 0.15 mg/mL, was incubated in the absence and presence of 4.1 (about 30 μg/mL) with varying amounts of spectrin dimer (specific activity in the region of 2500 c.p.m./μg) in 0.1 M sodium chloride, 30 mM Tris, 0.1 mM magnesium chloride, 0.1 mM ATP, and 0.5 mM dithiothreitol, pH 8.0. After 40 or 90 min at 20 °C, 200-μL aliquots were centrifuged at 80000*g* for 30 min, with or without a 10% sucrose cushion in the same buffer. The supernatants were carefully removed by aspiration, and the pellets, comprising not more than about 2% of the total assay volume, were counted directly in a Nuclear Chicago γ-radiation counter. Blanks, containing 4.1 and spectrin but no actin, were included in the assay. The amount of 4.1 in the pellet was determined by gel electrophoresis as above and densitometry. Approximate concentrations of the 4.1 preparations were obtained by the method of Bradford (1976), and spectrin and actin were standardized spectrophotometrically. It was ascertained by column chromatography on Sepharose 4B that on the time scale of the experiment no detectable conversion of spectrin dimer to tetramer took place.

Deoxyribonuclease Assays. The deoxyribonuclease (DNase) assay was used to follow the appearance of complexed, unavailable, actin with increasing proportions of spectrin dimer and 4.1. The procedure of Blikstad et al. (1978) was followed, whereby the F-actin-containing protein mixture

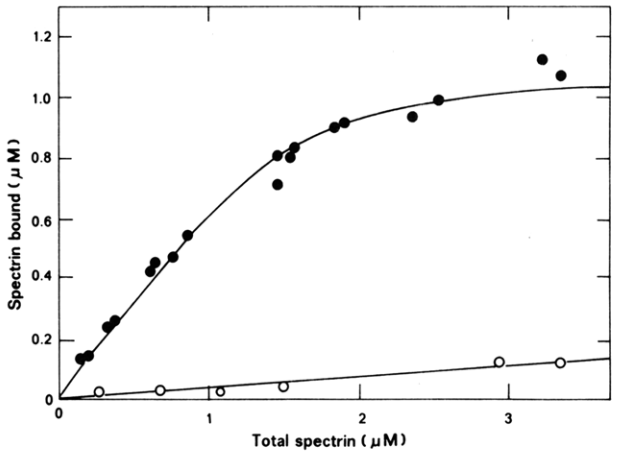

FIGURE 2: Binding of spectrin dimers to F-actin in the presence (●) and absence (○) of 4.1, at 20 °C and physiological ionic strength. The actin concentration was 10.1 μM and 4.1 (active) 1.1 μM. The curves are calculated best fits with an association constant of 4×10^3 M^{-1} for the binary system and a ternary association constant of 1.2×10^{12} M^{-2} for the upper curve. (For details of conditions and calculation, see text.)

was treated with 0.75 M guanidine hydrochloride to dissociate F-actin to monomers, which are capable of inhibiting the DNase activity. Complexes were prepared from G-actin, spectrin dimers, and 4.1, which were mixed in the required proportions. The solution was then made 0.1 M in sodium chloride and 1 mM in magnesium chloride and allowed to come to equilibrium for 3 h at room temperature, followed by 20 h at 4 °C. Assays were then carried out with column-purified deoxyribonuclease (Pinder & Gratzer, 1983) for both G-actin and available F-actin (Blikstad et al., 1978). To assay unavailable actin in the red cell membrane skeleton, proteolysis was used to destroy spectrin and 4.1 under conditions in which actin remains undamaged (Pinder & Gratzer, 1983). For this purpose aliquots of the complexes were incubated for 5 min at room temperature with 4 μg/mL TPCK–trypsin;[1] the reaction was stopped by addition of excess soyabean trypsin inhibitor, and available actin was again determined with DNase after exposure to guanidine hydrochloride.

Results

Binding of spectrin dimers and 4.1 to F-actin was measured by incubating the mixture of proteins at the desired temperature and pelleting the actin. The pellets were analyzed by γ counting for the content of radioiodinated spectrin and for 4.1 and actin by gel electrophoresis and densitometry. Equilibrium of binding was reached within 1 h. That errors due to dissociation of the complex during sedimentation could be ruled out was demonstrated by reincubating the complex with a 10-fold excess of unlabeled spectrin: after 120 min no radioactivity was released, as determined by analysis of the supernatant after pelleting.

The binding of spectrin dimers to F-actin at physiological ionic strength was found to be weak, with a binding constant at 20 °C of about 5×10^3 M^{-1} (Figure 2). At lower temperature binding was still weaker, and at 0 °C it was barely detectable as judged by the amount of spectrin carried down with the F-actin. The ionic strength dependence of binding is shown in Figure 3: possibly by virtue of nonspecific elec-

[1] Abbreviations: TPCK, tosylphenylalanine chloromethyl ketone; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS, sodium dodecyl sulfate.

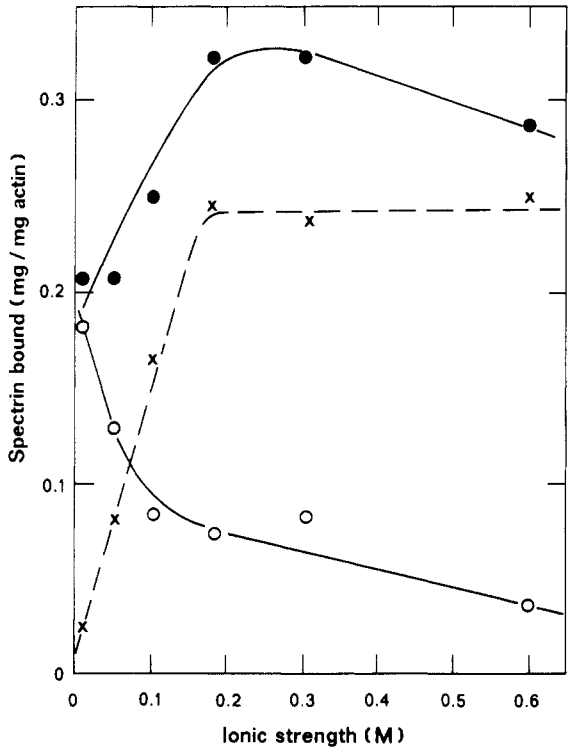

FIGURE 3: Ionic strength dependence of binding of spectrin dimers to F-actin at 20 °C in the presence (●) and absence (○) of 4.1. The broken line is the difference between the above, i.e., the excess spectrin bound when 4.1 is present. The ionic strength was adjusted with sodium chloride.

trostatic interactions binding increases markedly at low salt concentrations; between about 0.15 and 0.3 M it remains relatively constant.

When 4.1 was introduced into the system, binding of spectrin dimer was greatly enhanced (Figure 2). It was established that the amount of spectrin brought down with the actin was unchanged within the limits of detection when the radioiodinated protein was diluted with unlabeled protein, showing that iodination did not affect the affinity. The concentration of 4.1 was kept constant in these experiments.

By contrast with the binary spectrin–actin interaction, the ternary interaction in the presence of 4.1 showed reduced binding at low ionic strength with a plateau in the region above physiological (Figure 3). The pH dependence of binding was studied and showed a broad maximum in the physiological range. Quantitative analyses were carried out on results obtained at physiological salt concentrations and pH and in most cases at 20 °C. It is striking that, unlike the binary spectrin–actin complexes, the stability of this ternary complex showed no appreciable temperature dependence between 0 and 20 °C. No measurements could be made at higher temperatures because of partial conversion of spectrin dimers to tetramers (Ungewickell & Gratzer, 1978).

The binding profile (Figure 2) approaches a plateau with increasing total spectrin concentration, at a level corresponding to utilization of all the 4.1 in the system. The continuing slow rise in binding may be taken to correspond to the weak binding of spectrin to F-actin alone. Gel electrophoresis of the pellets of complex recovered at spectrin:actin ratios near the inflection of the binding profile showed that the mole ratio of spectrin:4.1 was 1.1 ± 0.3.

We were unable in these experiments to approach saturation of the actin by spectrin, because of limitations on the 4.1 concentrations that could be obtained; attempts at concentrating it further led to aggregation. At the same time the actin concentration had to be kept above the critical value (about 10 μg/mL under the conditions of these experiments). We cannot exclude the possibility that maximal binding corresponds to a spectrin:actin subunit ratio of less than unity, as a result, for example, of a crowding effect. Indeed in the titrations performed by DNase assays (Figure 4) the inflection point always appears to lie below equimolar (but see Discussion).

In attempting to analyze the data quantitatively, the concentration of 4.1, because of its tendency to become inactivated, is treated as an unknwon. We disregard the interaction between 4.1 and actin alone, since the evidence is that this is vanishingly weak in the absence of spectrin. In fitting the data we take account of the following equilibrium scheme, in which A represents an actin subunit, S the spectrin dimer, and F the 4.1:

$$A + S \underset{}{\overset{K_1}{\rightleftharpoons}} AS \qquad AS + F \underset{}{\overset{K_4}{\rightleftharpoons}} ASF$$

$$S + F \underset{}{\overset{K_2}{\rightleftharpoons}} SF \qquad SF + A \underset{}{\overset{K_3}{\rightleftharpoons}} ASF$$

The free energy of formation of the ternary complex, ASF, corresponds to the termolecular association constant for the interaction

$$A + S + F \rightleftharpoons ASF \qquad K_0\ (M^{-2})$$

$$K_1K_4 = K_2K_3 \equiv K_0$$

In the pelleting experiments we measure the total concentration of spectrin carried down on the F-actin, i.e., [ASF] + [AS] ≡ D (say). Then, writing for the input concentrations of spectrin, actin, and 4.1 $\bar{S}$, $\bar{A}$, and $\bar{F}$, respectively, and denoting the concentrations of the free components by a, s, and f, we have

$$\bar{A} = a + D$$

$$\bar{F} = f + [FS] + D - [AS]$$

$$\bar{S} = s + [FS] + D$$

$$K_1 = \frac{[AS]}{as}$$

$$K_2 = \frac{[FS]}{fs}$$

$$K_0 = \frac{D - [AS]}{asf}$$

We can eliminate [AS], [FS], a, f, and s from these equations and obtain an expression relating $\bar{A}$, $\bar{F}$, $\bar{S}$, and K_0, with K_1 and K_2 being taken respectively as 5×10^3 M^{-1} (from the data of Figure 1) and 5×10^6 M^{-1} (Wolfe et al., 1982). This yields the following expression, which is quadratic in $\bar{S}$:

$$\bar{S}^2\mu(\mu\lambda + K_2) - \bar{S}[\nu(2\mu\lambda + K_2) + \mu(K_2\bar{F} + 1)] + \nu(\nu\lambda + K_2\bar{F} + 1) + D = 0$$

Here

$$\lambda \equiv K_2[K_1(\bar{A} - D) + 1]$$

$$\mu \equiv \frac{K_0}{K_0 - K_1K_2}$$

$$\nu \equiv \frac{K_2D + K_0(\bar{A} - D)D}{(\bar{A} - D)(K_1K_2 - K_0)}$$

The data were fitted by computer to give the best values for K_0 and $\bar{F}$. The value of $\bar{F}$ (1.1 μM for the experiment depicted in Figure 1) was in good accord with the estimated active concentration (1.03 μM); K_0 was found to be 1.2×10^{12} M^{-2}, corresponding to an association free energy of 70 kJ mol^{-1},

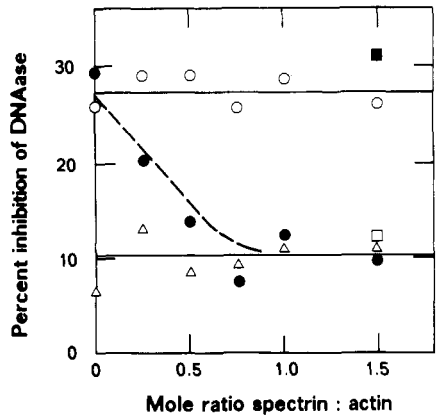

FIGURE 4: Deoxyribonuclease inhibition assay on ternary complexes between actin, spectrin dimers, and 4.1. The actin concentration was kept constant, and increasing proportions of spectrin and 4.1 were added before polymerization. Assays were performed (a) before treatment with 0.75 M guanidine hydrochloride (Blikstad et al., 1978), measuring essentially the critical monomer concentration (△), (b) after treatment with guanidine hydrochloride (●), when they measure the proportion of actin not refractory to dissociation, and (c) after trypsin treatment to destroy the spectrin and 4.1 selectively, followed by guanidine hydrochloride (○). The other two measurements show the inhibition with (■) and without (□) guanidine hydrochloride treatment in the binary system, containing only actin and spectrin dimers.

compared to about 20 kJ mol^{-1} for the binary complex of spectrin and actin. We have performed extensive tests in both our laboratories for effects of calcium ions on the binding equilibrium using EGTA buffers to give free calcium ion concentrations between about 1 nM and 1 mM. No calcium dependence was observed.

Figure 4 shows that the formation of the ternary complex is accompanied by sequestration of actin, which becomes unavailable for DNase assay after exposure to standard depolymerizing conditions. In this it resembles an important part of the actin of the membrane skeletal complex in the cell (Pinder & Gratzer, 1983). As in the latter, the actin becomes available after tryptic degradation of the spectrin and 4.1, which leaves the actin intact. No refractory state is detected when spectrin dimer is added to actin in the absence of 4.1.

Discussion

We have found that the association in vitro of the three major proteins of the red cell membrane skeleton leads to a tight complex, resembling the native membrane skeleton in the characteristic resistance of the actin to dissociating conditions (Pinder & Gratzer, 1983). The system is probably rather complex; for it appears from electron microscopy (Cohen et al., 1980) and actin nucleation experiments (Husain et al., 1983; Pinder et al., 1984) that spectrin and 4.1 have a high affinity for the ends of F-actin filaments and possess some degree of filament breaking activity. Thus, two types of complex may become important when the ratio of spectrin to actin subunits is high, depending also on the actin concentration. Inferences from such experiments (Figure 4) regarding the stability and stoichiometry of the complex therefore require caution. The binding profiles obtained by pelleting, under conditions in which the actin is in large excess and the strong binding is limited by the concentration of 4.1, should be much less affected by such considerations; this indeed is clear from the fact that the actin in the ternary complex pellets under these conditions like free F-actin.

The binding profiles shown in Figure 2 confirm that the binding of spectrin dimer to actin is very weak. This is qualitatively in accord with the data of Brenner & Korn (1979), who observed the association of sheep spectrin with actin at intermediate ionic strength and obtained curves consistent with an association constant of the order of 10^4 M^{-1}. Protein 4.1 causes greatly enhanced binding of spectrin to actin, as has been qualitatively demonstrated for the proteins in solution (Ungewickell et al., 1979; Fowler & Taylor, 1980), as well as association of F-actin with membrane vesicles (Cohen & Foley, 1982).

The total lack of calcium sensitivity of ternary complex formation stands in contrast to the calcium-dependent viscosity changes in complexes formed from G-actin (polymerized in the mixture), spectrin, and 4.1 (Fowler & Taylor, 1980) and the thixotropy of gels generated by such mixtures. We can conjecture only that these effects may have arisen from the effect of calcium on the actin polymerization per se (Borejdo et al., 1981; Maruyama, 1981).

In extracting an equilibrium constant for the ternary association some assumptions are implied. One is that the sites on the F-actin for spectrin–4.1 complex are independent. In earlier experiments from one of our laboratories (Wolfe et al., 1982) using 4.1 prepared by a different means, sigmoidicity was observed in the binding curves. For all data obtained subsequently in both our laboratories with fresh preparations of 4.1 made by improved procedures, no cooperative effects could be detected. The most likely basis for the appearance of cooperativity in this system is the propensity of 4.1 to self-associate (Ungewickell et al., 1979). Second, equimolar stoichiometry is assumed for the complex; as indicated, this is not altogether securely established, but it has little effect on the extracted binding constant. Even if the complex contained two actin subunits per spectrin, the binding constant would be reduced by only a factor of 2 and the corresponding negative binding free energy by less than 2 kJ mol^{-1}, with an effectively identical fit to the experimental points.

The association constant for the formation of a complex between 4.1 and spectrin dimer has been determined to be 5×10^6 M^{-1} (Wolfe et al., 1982) or 1×10^7 M^{-1} (Tyler et al., 1980). This corresponds to a standard free energy of some −40 kJ mol^{-1}, which accounts for a sizable part of the total standard free energy change of about −70 kJ mol^{-1} for formation of the ternary complex, and the spectrin–actin interaction free energy (−20 kJ mol^{-1}) accounts for much of the rest. The remainder may arise simply from a favorable cratic entropy increment because two interactions are satisfied by one ternary complex. On the other hand, an "allosteric" mechanism, whereby the spectrin undergoes a local conformational change on binding one or the other of its partners and thus gives an advantage that can be expressed as a coupling free energy (Weber, 1972), cannot be excluded. This coupling free energy would have a value of about −10 kJ mol^{-1}. It should be borne in mind that for spectrin tetramers, which are divalent with respect to actin, much stronger binding is to be expected on entropic grounds.

It may be stated finally that interactions of the type that we have studied are likely to be equally relevant to other cytoskeletal systems that contain actin and analogues of spectrin and 4.1 (Lazarides & Nelson, 1982; Baines, 1983).

Acknowledgments

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Identification of a Protein Kinase as an Intrinsic Component of Rat Liver Coated Vesicles†

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ABSTRACT: Purified rat liver coated vesicles phosphorylate two peptides, M_r 53 000 and M_r 51 000, in the presence of [γ-^{32}P]ATP. Incorporation of phosphate into these peptides is not stimulated by cAMP, Ca^{2+}, or Ca^{2+} plus calmodulin and occurs principally on a threonine residue. Mild conditions that result in removal of coat proteins from coated vesicles remove most of the protein kinase activity, suggesting the enzyme(s) is (are) not an integral membrane protein. Photolabeling of coated vesicles with 8-azido-[α-^{32}P]ATP results in specific labeling of only the M_r 53000 and M_r 51000 peptides. Preincubation with 10 mM *N*-ethylmaleimide inhibits kinase activity and concomitantly reduces photolabeling of the two peptides. Thus, the data are consistent with the hypothesis that protein kinase activity resides with these two coated vesicle proteins and that they are catalyzing an autophosphorylation reaction.

Coated vesicles are cagelike structures that function in eucaryotic cells to transfer membrane material to and from various subcellular structures (Goldstein et al., 1979; Pearse & Bretscher, 1981). Coated vesicles consist of membrane encapsulated by a protein coat. Coated vesicles are comprised principally (ca. 70%) of an M_r 180 000 protein called clathrin (Pearse, 1975) as well as several families of polypeptides whose molecular weights cluster respectively at 100 000, 50 000–55 000, and 34 000 (Blitz et al., 1977; Pearse, 1978; Woodward & Roth, 1978; Pfeffer & Kelly, 1981). Coated vesicles arise from coated regions of membranes usually referred to as coated pits, when they occur at the cell surface. The coated pits invaginate and assume an icosahedral surface to form coated vesicles. The coat proteins are rapidly lost at this point with the resultant formation of smooth vesicles. There is evidence that uncoating requires ATP and a specific uncoating enzyme (Patzer et al., 1982). There are minimal data on what factors govern, in vivo, formation of a coated pit, its evolution into a coated vesicle, and its movement within a cell. In vitro, assembly of coat structures occurs spontaneously from purified

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