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Regulation of intracellular levels of calmodulin and tubulin in normal and transformed cells

(calcium/calcium-binding proteins/cytoskeleton)

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ABSTRACT **Transformation of mammalian tissue culture cells by oncogenic viruses results in a 2-fold increase in the intracellular concentration of calmodulin quantitated by radioimmunoassay. The two pairs of companion cell lines used in this study were the Swiss mouse 3T3/simian virus 40-transformed 3T3 cells and the normal rat kidney (NRK)/Rous sarcoma virus-transformed NRK cells. The increased intracellular levels of calmodulin in the transformed cells are due to a greater increase in the rate of synthesis (3-fold) relative to the change in the rate of degradation (1.4-fold). On the other hand, no increases were observed in tubulin levels as quantitated by a colchicine-binding assay. The lack of change in tubulin concentration was accounted for by a 2-fold increase in the rate of degradation that is compensated by a similar increase in the rate of synthesis. The consequence of such changes in both transformed cell types is a 2-fold increase in the calmodulin-to-tubulin protein ratio relative to that in their nontransformed counterparts.**

Calmodulin is the major Ca^{2+}-binding protein in smooth and nonmuscle cells and has been proposed to represent an intracellular receptor for Ca^{2+} (1). This protein is ubiquitous, structurally conserved, and involved in the regulation of a variety of fundamental intracellular systems that control a large number of cellular activities (for review see refs. 1–3). For these reasons, many investigators have suggested that the activity of calmodulin would be primarily regulated by alterations in the net flux or distribution of Ca^{2+} and not by changes in the intracellular concentration of the protein. However, increases in the amount of calmodulin relative to total protein have been reported in chicken embryo fibroblasts transformed by Rous sarcoma virus, with calmodulin levels quantitated by either densitometric scanning of polyacrylamide gels (4) or bioassay (5). While both reports demonstrated at least 2-fold increases in the calmodulin specific activity in the transformed cells, neither study determined whether these values represented a true increase in the total intracellular levels of this protein. The present study was undertaken in order to determine whether such changes represented a more general response to transformation by oncogenic viruses and whether an increase in specific activity actually represented an increase in the number of calmodulin molecules per cell. Two cell systems were chosen for these studies: Swiss mouse 3T3 cells and their counterparts transformed by a simian DNA virus, SV40 and normal rat kidney (NRK) cells and those cells transformed by the Rous sarcoma RNA virus. Because the intracellular level of a protein is a function of its relative rates of synthesis and degradation, these parameters were determined in 3T3/SV40 3T3 cells. Tubulin levels have been previously reported not to increase after transformation (6), but the state of microtubule polymerization is regulated by Ca^{2+}-calmodulin (7, 8). Therefore, similar experiments were carried out on tubulin in order to evaluate the consequence of increased calmodulin levels on one system regulated by this protein. We demonstrate that transformation by oncogenic viruses results in an increase in the number of calmodulin molecules per cell by preferentially stimulating the synthesis of this protein, whereas no such elevation was observed for tubulin. The potential importance of the resultant change in the calmodulin-to-tubulin ratio is evaluated.

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EXPERIMENTAL PROCEDURES

Materials. All components of the tissue culture media were of the highest purity available and purchased from Sigma. Heat-inactivated fetal calf serum was purchased from GIBCO. Pansorbin was purchased from Calbiochem-Behring. L-[^{35}S]Methionine (1200 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) and ^{125}I-labeled *N*-succinimidyl-3-(4-hydroxyphenyl)propionate (^{125}I-labeled Bolton–Hunter reagent) were purchased from Amersham.

Tissue Culture. Normal Swiss 3T3 and the SV40-transformed Swiss 3T3 cell line (SV-3T3) as well as NRK cells and the Rous sarcoma virus-transformed NRK cell line (SR-NRK) were maintained in culture as described (9, 10).

Incorporation of [^{35}S]Methionine. One day prior to the introduction of [^{35}S]methionine, 3T3 and SV-3T3 cells were replica plated onto a series of 100-mm Falcon tissue culture petri dishes so as to ensure that cells would be in exponential growth for the duration of the experiments. On the following day, the normal growth medium [Dulbecco's minimal essential medium supplemented with 10% fetal calf serum, DME medium (+)] was removed, and the cultures were washed three times with 10 ml of Dulbecco's phosphate-buffered saline (P_i/NaCl) and incubated at 37°C for 30 min with medium devoid of methionine and fetal calf serum [DME medium (−)]. The cultures were then washed three times with P_i/NaCl before DME medium (−) containing [^{35}S]methionine at 100 μCi per plate was added. The cultures were incubated at 37°C and dishes were removed for analysis every 30 min.

Pulse–Chase. One day prior to the experiment, 3T3 and SV-3T3 cells were replica plated in a manner to ensure that cells would be in exponential growth throughout the experiment. On the following day the cultures were washed three times with 10

Abbreviations: SV40, simian virus 40; NRK cells, normal rat kidney cells; DME medium (+) and DME medium (−), Dulbecco's minimal essential medium supplemented with 10% fetal calf serum or devoid of methionine and fetal calf serum, respectively; P_i/NaCl, phosphate-buffered saline; PSDT buffer, phosphate/saline/deoxycholate/Triton X-100 buffer [contains methionine and ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid].

ml of P_i/NaCl, then incubated with DME medium (−) for 30 min at 37°C. The cultures were then washed three times in P_i/NaCl and incubated for 2.5 hr in DME medium (−) containing [^{35}S]methionine at 100 μCi per plate. After this pulse, the cultures were washed three times with P_i/NaCl containing unlabeled methionine at 100 μM (a 10^4 molar excess). DME medium (+) was then added to the cultures and they were incubated at 37°C for the next 30 hr, during which dishes were periodically removed for analysis.

Preparation of Tissue Culture Cells. At the appropriate time points, cultures labeled with [^{35}S]methionine were washed with 50 ml of P_i/NaCl containing 100 μM unlabeled methionine at 4°C. The cells were then removed from the dishes by scraping with a rubber policeman. The cells were centrifuged in a Beckman Microfuge for 2 min and the pellet was washed three times with P_i/NaCl containing 100 μM methionine. The final cell pellet was resuspended in 1 ml of 10 mM sodium phosphate/150 mM NaCl/1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetracetic acid (EGTA)/100 μM methionine/1% Triton X-100/1% deoxycholate, pH 7.5 (PSDT buffer), at 4°C and homogenized by sonication with a Brownwill Biosonik IV sonicator. Portions were removed from each sample for the determination of total protein concentrations by using the Bradford procedure (11) with gamma globulin as standard. The remaining volume from each sample was then divided into two equal aliquots. One of the aliquots was subject to heat treatment at 90°C for 5 min followed by rapid cooling in an ice bath, while the other half remained untreated. All samples were then centrifuged at 10,000 × *g* for 30 min at 4°C, and the supernatant solutions were removed for analysis. Total calmodulin levels were determined by radioimmunoassay as described (12), whereas total tubulin levels were determined by colchicine binding assay as described by Wilson and Bryan (13).

Quantitation of [^{35}S]Methionine-Labeled Proteins. Total protein labeled with [^{35}S]methionine was determined by incubating 10 μl of each of the non-heat-treated samples with 5 ml of 10% trichloroacetic acid at 4°C for 30 min. The acid-precipitable radioactivity was recovered on glass fiber filters. The filters were then washed three times with 10 ml of 10% trichloroacetic acid at 4°C followed by one wash with 95% (vol/vol) ethanol and one wash with chloroform. The filters were dried and radioactivity was determined in a Beckman scintillation counter, using Spectrofluor (Beckman) scintillation cocktail.

[^{35}S]Methionine-labeled calmodulin was immunoprecipitated by a monospecific sheep antibody to calmodulin (12). One hundred microliters of affinity-purified anti-calmodulin (1 mg/ml) in PSDT buffer was added to 100 μl of each heat-treated supernatant solution, to which 1000 cpm of authentic ^{125}I-labeled calmodulin (12) had been added to correct for recovery. Nonspecific binding was determined by substituting 100 μl of preimmune IgG for anti-calmodulin. All samples were incubated at 25°C for 18 hr, after which time 25 μl of a 10% solution of Pansorbin was added and incubated with the samples for 30 min at 25°C. After this incubation each suspension was layered over 500 μl of 1 M sucrose in PSDT and centrifuged in a Beckman Microfuge for 2 min. The supernatant fluid was carefully removed and pellets were washed with PSDT buffer. The ^{125}I in the final pellet was measured in a Searle gamma counter in order to determine the percent recovery of calmodulin. The pellets were resuspended in 100 μl of 60 mM Tris·HCl/7 mM 2-mercaptoethanol/3% sodium dodecyl sulfate, pH 6.8, and heated for 5 min at 95°C. The samples were cooled and [^{35}S]methionine was measured in a Beckman scintillation counter, using BBS-3 (Beckman) scintillation cocktail. The immunoprecipitation of [^{35}S]methionine-labeled tubulin was accomplished in an identical manner, using a monospecific antibody to tubulin. This antibody was prepared in rabbit as reported by Fuller *et al.* (14).

Each experiment reported in this manuscript was repeated three times and each assay was performed in triplicate. In all cases the intra-assay variability was less than 3%. The absolute values obtained in the three experiments varied due to the differences in the age and passage number of the cells utilized, although the trends and ratios were the same in each case. Because of these differences, the data were not treated statistically with respect to interassay variability.

RESULTS

As shown in Fig. 1, both 3T3 and SV-3T3 cells exhibited linear incorporation of [^{35}S]methionine into total protein, calmodulin, and tubulin for at least 4 hr. In all cases the rate of incorporation of [^{35}S]methionine into protein was significantly greater in the SV40-transformed 3T3 cells. Whereas total protein and tubulin were synthesized at a rate approximately 2 times greater in the transformed cells, calmodulin was synthesized at a rate 3 times more rapid.

A comparison of the relative rates of degradation for total protein, calmodulin, and tubulin in 3T3 and SV-3T3 cells is presented in Fig. 2 as total radioactivity incorporated per plate. In all three cases exponential rates of decay were observed during

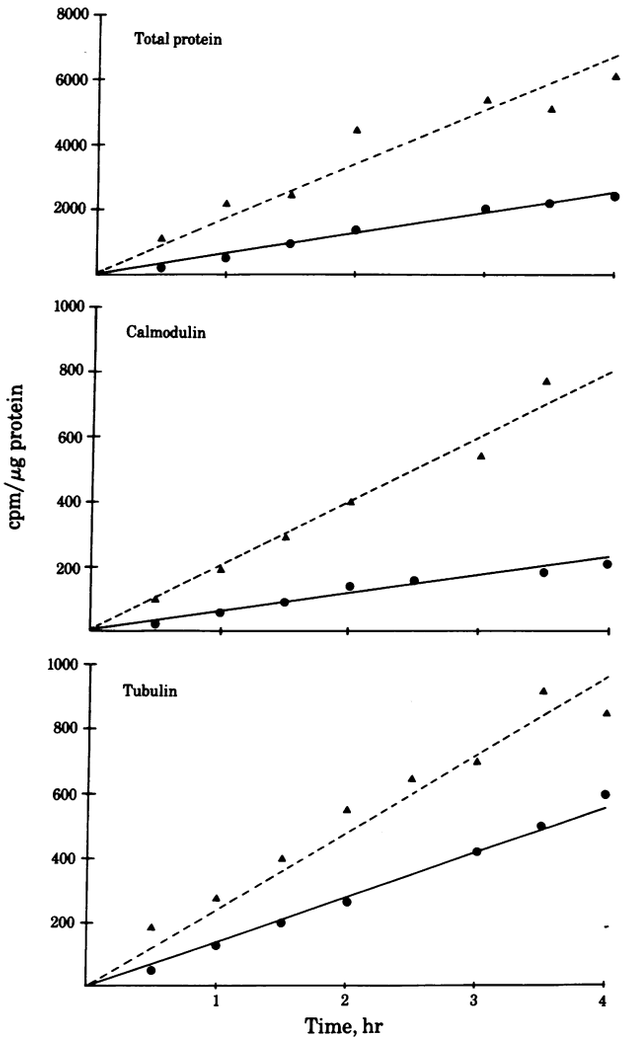

FIG. 1. Incorporation of [^{35}S]methionine into total protein, calmodulin, and tubulin by 3T3 (●) and SV-3T3 (▲) cells.

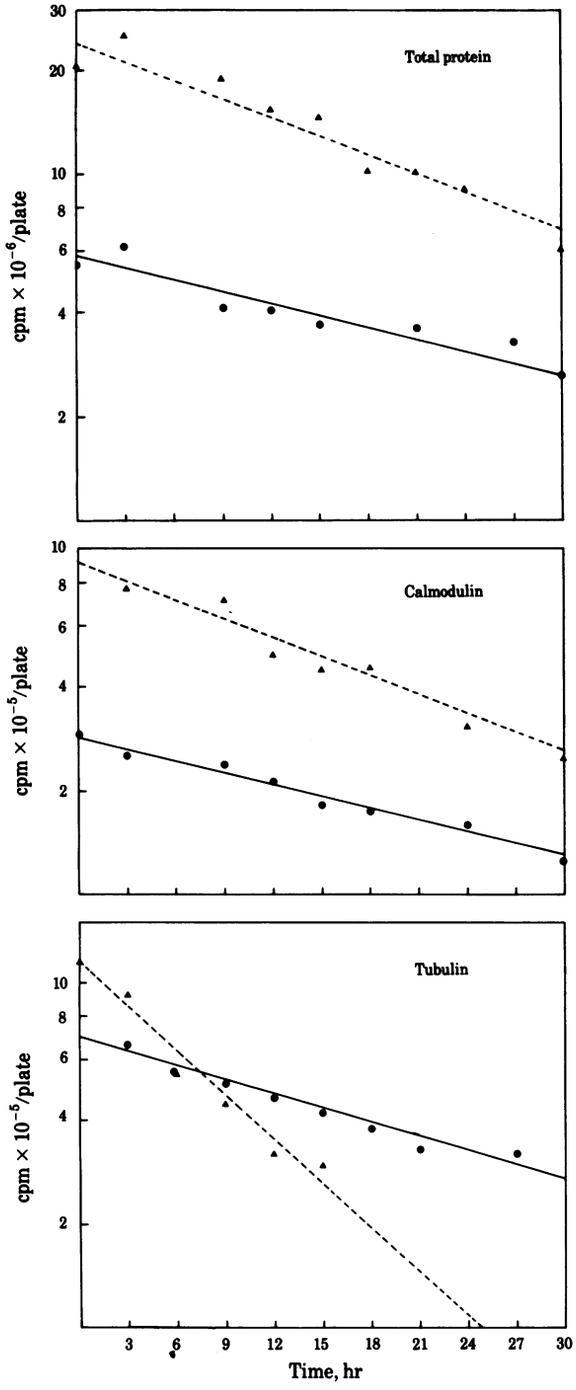

FIG. 2. Relative rates of degradation for [^{35}S]methionine-labeled total protein, calmodulin, and tubulin in 3T3 (●) and SV-3T3 (▲) cells.

the 30-hr period after the addition of 10^4 molar excess unlabeled methionine. In addition, the relative differences of [^{35}S]methionine incorporation observed between the 3T3 and SV-3T3 cells at time zero are consistent with the differential incorporation of the labeled amino acid observed in Fig. 1. In all cases the rate of protein degradation appears to be more rapid in the SV-3T3 cells than in their nontransformed counterparts. Total protein exhibits an apparent half-life $t_{1/2}$, of 26 hr in the 3T3 cells and 20 hr in the SV-3T3 cells. Calmodulin changes in a manner similar to total protein, with apparent $t_{1/2}$ values of 25 and 18 hr, respectively, for the normal and transformed cells. In contrast, tubulin exhibits a greater change in $t_{1/2}$ in transformed cells. Thus 3T3 cells yield an apparent $t_{1/2}$ of 24 hr compared to 10 hr for the SV-3T3 cells.

Exponentially growing cells maintain intracellular protein concentrations at steady-state levels. Steady-state protein concentrations in 3T3 and SV-3T3 cells may be expressed by the following relationship (15):

$$[\text{protein}]_{ss} = k_s/k_d,$$

in which $[\text{protein}]_{ss}$ is the steady-state concentration of a specific protein in ng per μg of total protein, k_d is the rate of degradation in hr^{-1}, and k_s is the rate of synthesis for that protein in pg min^{-1} per μg of total protein. Each of these three variables was independently measured in two cell types. Steady-state protein concentrations were measured by the appropriate assays, k_s was measured as the slope of the curves in Fig. 1, and k_d was generated from Fig. 2 by using the equation $t_{1/2} = (\ln 2)/k_d$ (15). The validity of each independent measurement was verified by using the experimentally obtained rates of synthesis and degradation to generate a calculated steady-state protein concentration. As shown in Table 1, the experimental values for calmodulin, tubulin, and total protein are in close agreement to the mathematically derived values in all cases. These data also demonstrate that the steady-state concentration of tubulin is maintained at the same level in both the normal and the transformed cells through a change in its rate of degradation that is commensurate with the change in its rate of synthesis. The steady-state concentration for calmodulin, however, is increased in the transformed cell through an increase in its rate of synthesis that is greater than the change in its rate of degradation.

In order to determine whether the relationship between steady-state protein concentrations and cell transformation was unique to the 3T3/SV-3T3 system, the relative differences in the intracellular steady-state protein concentrations in the NRK/SR-NRK system were also measured. This comparison is illustrated in Table 2. Intracellular tubulin levels are only slightly increased in the corresponding transformed cells of both systems. This increase is identical to that observed for total protein. However, intracellular calmodulin levels are more than doubled in both types of transformed cells. Together, these data reveal that there is a 2-fold increase in the molar ratio of calmodulin to tubulin in both transformed cell types.

DISCUSSION

We report in this communication that total intracellular levels of calmodulin are increased in mammalian tissue culture cells transformed by DNA or RNA viruses. In the case of SV40-mediated transformation, this increase appears to be due to a selective increase in the rate of synthesis of this protein in the transformed cells. These findings support and extend earlier studies describing increased activities of calmodulin in a single cell type (chicken embryo fibroblasts) transformed by an RNA virus (Rous sarcoma) (4, 5). Our data suggest that increase in the intracellular levels of calmodulin may be common to transformation of eukaryotic cells mediated by oncogenic viruses. The potential importance of such an increase in calmodulin levels is suggested by the fundamental regulatory roles this protein plays in all eukaryotic cells.

Calmodulin is a multifunctional intracellular Ca^{2+}-binding protein involved in the regulation of such essential intracellular processes as cyclic nucleotide and glycogen metabolism, Ca^{2+}-dependent protein phosphorylation, cell division, and other forms of motility (for reviews see refs. 1–3). The overall effects of such a multifunctional protein must be the result of a critical balance between all the regulated systems involved. The perturbation of the intracellular levels of calmodulin may well be

Table 1. Steady-state kinetics of calmodulin, tubulin, and total protein in 3T3 and SV-3T3 cells

Protein	Cells	$t_{1/2}$, hr	k_d,* hr^{-1}	k_s,† pg/min per μg protein	[Protein]$_{ss}$, ng/μg protein	
					Assay	Calc.‡
Calmodulin	3T3	25.5 ± 2.5	0.027	0.30	0.63	0.66
	SV-3T3	18 ± 1	0.039	0.90	1.33	1.38
Tubulin	3T3	24	0.029	15	33	32
	SV-3T3	10	0.070	34	31	30
Total protein	3T3	26	0.027	—	2300	—
	SV-3T3	20	0.038	—	2700	—

$t_{1/2}$ values for calmodulin are mean ± SEM for nine measurements.
* Calculated from Fig. 2 by using $k_d = (\ln 2)/t_{1/2}$.
† Calculated as the slope in Fig. 1.
‡ Calculated by using [protein]$_{ss} = k_s/k_d$.
§ Units are pg per cell.

expressed through alterations in those systems regulated by intracellular Ca^{2+}. Cell transformation is usually accompanied by alterations in morphology (16), cyclic nucleotide metabolism (17), metabolic rate (18), and intracellular Ca^{2+} levels (19). In addition, transformed cells lose the requirements for anchorage to substrate (20, 21) and calcium for cell proliferation (22, 23). The effect of viral transformation on one Ca^{2+}/calmodulin-regulated system, the cytoplasmic microtubule network, has been extensively investigated in a large number of laboratories [see Brinkley *et al.* (16) for review]. Studies employing indirect immunofluorescence microscopy have been interpreted to reveal that the microtubule network undergoes a rearrangement (16). On the other hand, quantitative measurements of cellular content of tubulin have failed to reveal any changes in total tubulin levels after transformation (6). Therefore, the validity of the use of immunofluorescence techniques to evaluate changes in the cytoplasmic microtubule complex in transformed cells has been questioned (6). However, the recent work of Rubin and Waren (24), using quantitative electron microscopy, supports the rearrangement hypothesis. These authors provide evidence that transformed cells contain only half the number of polymerized microtubules that their nontransformed counterparts do. Because total intracellular tubulin levels are essentially the same in both cell types (Table 2; ref. 6), one apparent effect of viral transformation may lie in the regulation of microtubule assembly.

We have previously reported that calmodulin mediates the calcium-dependent assembly–disassembly of microtubules *in vitro* (7). This observation has now been confirmed by other laboratories (8, 25). Recently, using a detergent-permeabilized cell system to study polymerization of microtubules from phosphocellulose-purified 6S tubulin, Brinkley *et al.* (26) have shown that SV-3T3 cells exhibit a markedly reduced capacity to initiate and elongate microtubules from cytoplasmic microtubule organizing centers in comparison to normal 3T3 cells. Moreover, addition of calmodulin to the normal 3T3 system caused an inhibition of microtubule assembly that resulted in a substantial reduction in the number and length of microtubules (1). This phenotype, then, resembled that of the transformed state as determined by immunofluorescence techniques (1). Together our studies suggest that the degree of polymerization of tubulin into microtubules *in vivo* may be dependent on the concentration of calmodulin within the cell relative to that of tubulin. Thus, the increased levels of calmodulin in transformed cells could destabilize microtubules and result in a significantly reduced number and length of microtubules in comparison to those observed in nontransformed cells. This possibility is supported in NRK cells, in which a 2-fold increase in intracellular levels of calmodulin relative to tubulin in the transformed cell (Table 2) appears to be accompanied by a 2-fold increase in the percent of depolymerized microtubules as quantitated by electron microscopy (24).

If calmodulin does affect the intracellular regulation of tubulin assembly, it could also be postulated to alter the intracellular levels of tubulin, due to the results of a report by Ben-Ze'er *et al.* (27). These investigators have proposed that an increase in the ratio of unpolymerized to polymerized tubulin increases the rate of degradation by affecting the turnover of tubulin mRNA. These observations suggest that an increase in unpolymerized tubulin in the transformed cell should result in an increase in the rate of degradation of that protein relative to what occurs in the nontransformed state. We have shown in this communication (Fig. 2) that such a situation does exist in 3T3 cells transformed by SV40. Thus calmodulin may alter the cytoskeleton not only through a direct effect on microtubule assembly but also indirectly through regulation of the intracellular levels of tubulin. It appears, therefore, that perturbation of the intracellular regulation of calmodulin concentration may be responsible, at least in part, for some of the phenotypic changes observed after viral transformation of some tissue culture cell lines. Our

Table 2. Comparison of calmodulin and tubulin concentrations in normal and transformed cells

Cells	Total protein		Tubulin		Calmodulin		Calmodulin/tubulin	
	pg/cell	Transformed/normal	pg/cell	Transformed/normal	pg/cell	Transformed/normal	Molar ratio	Transformed/normal
3T3	230	1.2	7.36	1.1	0.15	2.4	0.13	2.3
SV-3T3	280		8.32		0.36		0.30	
NRK	210	1.2	6.4	1.2	0.27	3.2	0.28	2.6
SR-NRK	260		7.9		0.88		0.72	

Total intracellular levels of total protein, tubulin, and calmodulin were determined in exponentially growing cells.

data also suggest that increased calmodulin levels may be a general response to cell transformation. Indeed, preliminary experiments have revealed a similar increase in mouse mammary epithelial tumor cells (unpublished observations). Our recent success in the cloning of a calmodulin structural gene probe (28) will allow subsequent studies to determine whether these alterations are due to increased levels of calmodulin mRNA.

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