and in the tumor cells derived from all tumors induced in nude mice, we conclude that no suppression of the malignant phenotype takes place in somatic cell hybrids between normal diploid mouse cells and SV40-transformed human cells.

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Retention of Nonhelical Procollagen Containing *cis*-Hydroxyproline in Rough Endoplasmic Reticulum

Abstract. *Fibroblasts freshly isolated from embryonic tendons were incubated with a proline analog,* cis*-4-hydroxy-*L*-proline, which is incorporated into protein and which leads to the intracellular accumulation of nonhelical procollagen. Evidence is presented here that the nonhelical procollagen containing the analog is retained within the rough endoplasmic reticulum and does not pass to the smooth endoplasmic reticulum or Golgi vacuoles at a normal rate.*

Collagen is first synthesized as a precursor molecule called procollagen, and assembly of procollagen involves several steps: (i) incorporation of amino acids into the three pro-α chains of procollagen; (ii) hydroxylation of some of the prolyl and lysyl residues in the newly synthesized polypeptides to hydroxyprolyl and hydroxylysyl residues and the subsequent glycosylation of some of the hydroxylysyl residues; (iii) association of the three pro-α chains largely through interactions among the globular, peptide extensions on the chains; (iv) folding of the collagen portions of the pro-α chains into the triple-helical conformation characteristic of collagen (*1, 2*). The triple-helical conformation is required for procollagen to be secreted at a normal rate (*1, 2*). The role of the triple-helical conformation in secretion has largely been established by experiments in which cells or tissues synthesizing procollagen are incubated without oxygen or with the iron chelator α,α'-dipyridyl under conditions that allow polypeptide synthesis to continue at about the control rate for several hours but which completely prevent the hydroxylation of proline and lysine (*3*). Under such conditions, cells accumulate the unhydroxylated form of procollagen, which has been called protocollagen and which cannot fold into a stable triple-helical conformation at physiological temperatures because it lacks hydroxyproline (*4*). In cells incubated with α,α'-dipyridyl, protocollagen accumulates within the cisternae of the rough endoplasmic reticulum (*5, 6*); this observation suggested that the triple-helical conformation is required for the protein to pass at a normal rate from the endoplasmic reticulum to the smooth endoplasmic reticulum and Golgi vacuoles. We now provide further evidence for this conclusion by incubating cells with a proline analog, *cis*-4-hydroxy-L-proline, which is incorporated into protein (*7*). Our experiments provide a chemically independent test for the effect of conformation on

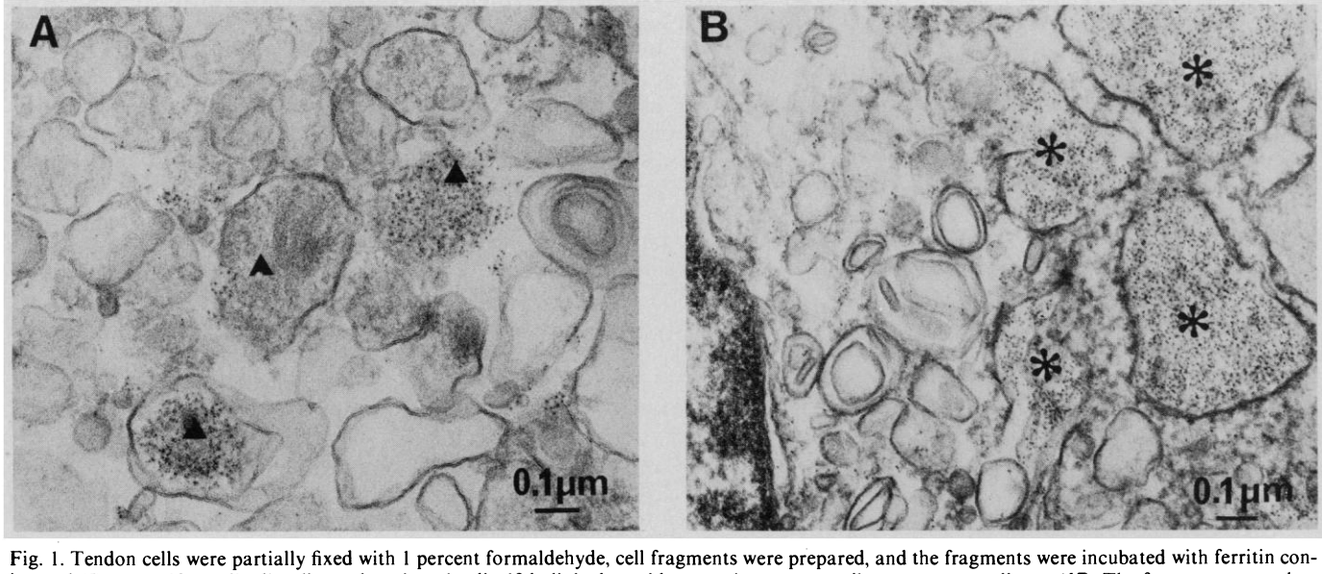

Fig. 1. Tendon cells were partially fixed with 1 percent formaldehyde, cell fragments were prepared, and the fragments were incubated with ferritin conjugated (*5*) to specific antibodies directed against the disulfide-linked peptide extensions on procollagen or protocollagen (*17*). The fragments were then washed, further fixed with 3 percent glutaraldehyde, and postfixed with 1 percent osmium tetroxide. The samples were stained with 1 percent uranyl acetate (Polysciences) for 180 minutes at room temperature, dehydrated with ethanol and acetone, and embedded in Epon 812 (Electron Microscopy Sciences) (*5*). Ultrathin sections were prepared and examined with a JEM-100B electron microscope (JEOL). (**A**) Fragments from cells incubated with 1 μM colchicine for 2 hours. Golgi vacuoles are large and many contain fibrillar material. Triangles indicate Golgi vacuoles labeled with ferritin-antibody conjugates. Cisternae of the rough endoplasmic reticulum were also labeled (not shown). (**B**) Fragment from cells incubated with 1 μM colchicine and 1.53 mM *cis*-hydroxyproline for 2 hours. Golgi vacuoles are small and not specifically labeled with the ferritin-antibody conjugates. Asterisks indicate cisternae of the rough endoplasmic reticulum which are extensively labeled with the conjugates.

intracellular transport of the protein, since newly synthesized procollagen polypeptides containing *cis*-hydroxyproline remain nonhelical (*8*) but differ from protocollagen polypeptides in that they have almost the same content of hydroxyproline (*9*), hydroxylysine, and glycosylated hydroxylysine (*10*) as procollagen.

Tendon fibroblasts were prepared by controlled enzymic digestion of leg tendons from 17-day-old chick embryos (*11*). These cells have been shown to synthesize and secrete procollagen at a rapid rate when incubated in suspension for up to 6 hours (*11*).

The cells were incubated with [^{14}C]proline and with 1 μM colchicine, 0.3 mM α,α'-dipyridyl, or 1.53 mM *cis*-4-hydroxy-L-proline for 120 minutes, and subcellular fractions were prepared with a procedure adapted for these cells (*12*). The procedure provides an effective isolation of a fraction containing smooth endoplasmic reticulum, but isolation of fractions containing rough endoplasmic reticulum is less satisfactory. In cells incubated under control conditions, about 23.7 percent of the total intracellular procollagen polypeptides were recovered in the smooth endoplasmic reticulum (Table 1). In cells treated with colchicine, the fraction of procollagen polypeptides recovered in the smooth endoplasmic reticulum increased to 45.6 percent. Incubation of the tendon cells with 1 μM colchicine delays secretion of procollagen (*11*) and produces distension of smooth endoplasmic reticulum and Golgi vacuoles (*5*). Therefore, the effect of colchicine seen here provides supportive evidence that the procedure for subcellular fractionation afforded effective isolation of smooth endoplasmic reticulum from other organelles. In cells incubated with α,α'-dipyridyl, the fraction of collagenous polypeptides recovered with the smooth endoplasmic reticulum decreased to less than half the control value, an observation consistent with previous indications that [^{14}C]protocollagen accumulates in the rough endoplasmic reticulum (*5, 6*). As noted here, incubation with *cis*-hydroxyproline decreased the amount of [^{14}C]procollagen in the smooth endoplasmic reticulum, even though the total intracellular [^{14}C]procollagen was slightly greater than in the control. The results indicated, therefore, that the intracellular distribution of nonhelical procollagen containing the analog was similar to [^{14}C]protocollagen. In the same series of experiments, incubation with either *cis*-hydroxyproline or with α,α'-dipyridyl completely inhibited colchicine-induced accumulation of ^{14}C-labeled protein in smooth endoplasmic reticulum.

Table 1. Tendon cells (3.3×10^8) were incubated at 37°C in 33 ml of modified Krebs medium containing 10 percent fetal calf serum with 1 μM colchicine (Sigma), 0.3 mM α,α'-dipyridyl (Eastman), or 1.53 mM *cis*-4-hydroxy-L-proline (Ajinomoto) (*11*). After 15 minutes, 10 μc of [^{14}C]proline (New England Nuclear) was added. Incubation was continued for 120 minutes, and was stopped by the addition of cycloheximide (100 μg/ml) and α,α'-dipyridyl (1 mM); the cells were recovered by centrifuging at 1200g for 10 minutes (*11*). The cells were washed once with modified Krebs medium containing cycloheximide and α,α'-dipyridyl, and they were then homogenized at 4°C in 3 ml of 0.25M sucrose, 25 mM KCl, 0.5 mM $MgCl_2$, and 50 mM tris-HCl, pH 7.5 (*12*). Homogenization was performed with 60 strokes in a Teflon and glass homogenizer driven at 1750 rev/min with a constant-torque motor (*5*). The homogenate was centrifuged at 15,000g for 15 minutes. The pellet and a portion of the supernatant were assayed for [^{14}C]procollagen or [^{14}C]protocollagen. About 1.5 ml of the supernatant was placed on a sucrose gradient prepared with the first buffer system of Harwood *et al.* (*12*). The first step was made with 2.5 ml of 0.5M sucrose, 0.5 mM $MgCl_2$, 25 mM KCl, and 50 mM tris-HCl, pH 7.5. The second step was made with 1 ml of 1.3M sucrose and the same salts and buffer. The sample was centrifuged (SW-50.1 Spinco head) at 33,800 rev/min (about 105,000g) for 16 hours. Fractions (0.5 ml) were collected and dialyzed against tap water. From 25 to 59 percent of the total nondialyzable ^{14}C in the gradient was recovered at the interface of the 0.5M and 1.3M sucrose, the region that was previously shown to contain smooth vacuoles as assayed by electron microscopy and enzyme markers (*12*). Abbreviation: dpm, disintegrations per minute.

Treatment of cells	Total cell content (10^4 dpm)		[^{14}C]Procollagen or [^{14}C]protocollagen* in smooth endoplasmic reticulum	
	^{14}C-labeled protein	[^{14}C]Procollagen or [^{14}C]protocollagen*	Amount (10^4 dpm)	Percent of total
Control	21.1	4.9	1.16	23.7
Colchicine	36.5	14.3	6.51	45.6
α,α'-Dipyridyl	24.8	15.4	0.55	3.6
cis-Hydroxyproline	19.1	5.8	0.21	3.6
cis-Hydroxyproline + colchicine	18.6	5.9	0.23	3.9
α,α'-Dipyridyl + colchicine	22.3	13.9	0.64	4.6

*[^{14}C]Procollagen polypeptides were assayed by a specific radiochemical assay for [^{14}C]hydroxyproline (*14*). The values for [^{14}C]hydroxyproline were corrected by a factor of 2, since [^{14}C]procollagen contains approximately equal amounts of proline and hydroxyproline (*15*). Similar results were obtained in other experiments in which [^{14}C]procollagen was assayed with a purified bacterial collagenase. In samples from cells that were incubated with α,α'-dipyridyl to inhibit prolyl and lysyl hydroxylases and which therefore contained [^{14}C]protocollagen, collagenous polypeptides were assayed by measuring the ^{14}C-labeled peptides which became dialyzable after the samples were incubated for 90 minutes at 37°C with purified bacterial collagenase (50 μg/ml) in 5 mM $CaCl_2$, 2.5 mM *N*-ethylmaleimide, and 50 mM tris-HCl buffer, pH 7.6. The bacterial collagenase (Sigma) was purified further by gel filtration (*16*).

In further experiments, cells were incubated as in Table 1, and cell fragments were prepared (*5*). Procollagen and protocollagen were then located in the cell fragments with ferritin conjugated to antibodies specific for the peptide extensions on both procollagen and protocollagen. Procollagen was readily located in the distended Golgi vacuoles which were seen in cells incubated with colchicine (*5*) (Fig. 1). In contrast, few large Golgi vacuoles were seen in cells treated either with α,α'-dipyridyl (*5*) or with α,α'-dipyridyl and colchicine (not shown). Also, there was no specific staining of smooth-membraned vacuoles with the ferritin-antibody conjugates. As indicated here, incubation with *cis*-hydroxyproline produced effects similar to α,α'-dipyridyl in that few large Golgi vacuoles were seen in the cells (Fig. 1). There was no specific labeling of vacuoles with the ferritin-antibody conjugates, even though the cisternae of the rough endoplasmic reticulum were readily stained.

Our results provide further and apparently conclusive evidence that freshly isolated tendon cells synthesizing procollagen contain a barrier or similar mechanism which can discriminate triple-helical from nonhelical forms of the protein, and that this discrimination occurs either within the cisternae of the rough endoplasmic reticulum or between this compartment and the smooth endoplasmic reticulum and Golgi vacuoles (*1, 13*). There is no direct evidence as to how this discrimination occurs (*1*), but at least three possibilities should probably be considered. (i) The cells contain a structural barrier such as membrane which allows only the triple-helical protein to pass at a normal rate. (ii) The triple-helical conformation confers on the molecule physical properties that are required for passage at an optimum rate through the secretory apparatus. (iii) The enzymes prolyl hydroxylase and lysyl hydroxylase serve as barriers in the sense that they bind the nonhelical form of the protein and thereby delay its secretion.

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Calcium Ion Distribution in Cytoplasm Visualized by Aequorin: Diffusion in Cytosol Restricted by Energized Sequestering

Abstract. *The distribution of Ca^{2+} in the cytoplasm following a local rise in Ca^{2+} concentration is visualized by means of aequorin luminescence and a television system with an image intensifier. Diffusion of Ca^{2+} through the cytosol is so constrained that a rise in cytoplasmic Ca^{2+} concentration produced by local Ca^{2+} entry through cell membrane or by local Ca^{2+} injection is confined to the immediate vicinity of these sites. The diffusion constraints are lifted by treatment with cyanide or ruthenium red. Thus, energized calcium sequestering, probably by mitochondria, is the dominant factor in the constraints. In cell regions where the sequestering machinery is sufficiently dense, different Ca^{2+} message functions inside a cell may be effectively segregated, permitting private-line intracellular communication.*

Among the cellular inorganic ions, Ca is rather unique in that it is rapidly sequestered by mitochondria (*1*). Probably largely because of this, a local increase in free cytoplasmic Ca^{2+} concentration, $[Ca^{2+}]_i$, such as produced by a Ca injection into a cell, falls off more steeply with distance than in simple diffusion (*2, 3*). We show here—in experiments in which the Ca distribution inside the cell is displayed by aequorin—that the spatial attenuation is so steep that a local increase in $[Ca^{2+}]_i$ in the cytosol is effectively compartmented.

Cells of isolated *Chironomus* salivary glands were injected with the Ca^{2+}-sensitive luminescent protein aequorin (*4*). The aequorin light emission, which increases with $[Ca^{2+}]_i$ (*5*), was viewed and recorded through a microscope with the aid of an image intensifier coupled to a TV camera (*6*) (Fig. 1, inset). The method has sensitivity for $10^{-6}M$ $[Ca^{2+}]_i$ over a 1-μm-diameter cytoplasmic volume (steady state), and a spatial resolution of 1 μm. The total luminescence was measured independently with a photomultiplier. Photomultiplier current, membrane potential, and Ca^{2+} injection current were displayed on a storage oscilloscope (and a chart recorder) onto which a second TV camera was focused. The two camera outputs were videotaped and displayed simultaneously on a TV screen. Test pulses of Ca^{2+} were introduced by iontophoresis into the basal region of the cells with micropipettes filled with $0.4M$ $CaCl_2$ and $0.2M$ ethylene glycol-bis(aminoethylether) tetraacetate (EGTA) (*p*H 7). The resulting $[Ca^{2+}]_i$ was estimated by deriving the unit-volume radiant fluxes in the (spherical) luminescent region and then matching these against equivalent fluxes produced by standard Ca-EGTA buffers. The buffers, aequorin, fluorescein, and ruthenium red were microinjected hydraulically.

Figure 1 illustrates an experiment in which a series of test Ca^{2+} pulses produced rather constant spherical aequorin glows. The striking feature is that the glows are confined to the vicinity of the micropipette tip. Evidently the rise in $[Ca^{2+}]_i$ does not extend much beyond this vicinity: the $[Ca^{2+}]_i$, which was $< 10^{-4}M$ within a radius of 16 ± 2 μm from the tip at the time of maximal glow spread, fell precipitously within the next 1 to 2 μm to $< 10^{-6}M$.

This Ca^{2+} confinement depends on energy metabolism. When the cell was poisoned with 2 m*M* cyanide (CN), the standard Ca^{2+} pulses produced glows which spread throughout the cytoplasm. The $[Ca^{2+}]_i$ then fell off more gradually with distance from the tip, resembling the pattern of a more freely diffusing molecule (Fig. 2). The glows then also lasted longer. At Ca^{2+} pulse repetition rates of 2.4 per minute, the glows initially faded out between pulses; but eventually they summed, as one might expect if the Ca^{2+} sequestering and extrusion capacities were depressed.

The CN effects were rapidly reversible upon washout of the poison. The CN treatment produced no detectable change in the background $[Ca^{2+}]_i$. This could be determined between earlier pulses (0 to 200 seconds; Fig. 1) in the test cell and throughout the experiment in the adjacent cell. However, at higher CN concentrations (5 m*M*), an increase in $[Ca^{2+}]_i$ due to intracellular Ca release was clearly visible.

In another set of experiments, the cells were injected with ruthenium red (0.1 m*M*), a blocker of mitochondrial Ca uptake (*7*). Here the glows of the Ca^{2+} test pulses became diffuse (Fig. 4, II) and lasted longer too.

The method was sensitive enough to detect minute inward leaks of Ca through cell membrane. Thus, for instance, while the microelectrodes were in the cells, leakage of Ca^{2+} from the medium could be seen at the regions of their insertion into the membrane. In medium containing $\geqslant 2$ m*M* Ca, such leakage was generally visible, even with electrodes with < 0.2-μm tip diameters, when cell input resistance and membrane potential were high (0.3 megohm and 30 to 40 mv). The leaks were seen as steady luminescent dots, sometimes as small as 1 μm in diameter, which were typically confined to the immediate vicinity of the membrane. As in the case of Ca injection, this confinement required metabolic energy. The glow spread upon treatment with 2 m*M* CN, eventually reaching deep into the cell, and after washout the glow returned to the original limits (Fig. 3). Here again the CN occasioned no significant internal Ca^{2+} release: upon change to a Ca-free CN medium at the peak of the glow spread, the glow ceased. The possibility of significant outward leakage of aequorin (molecular weight, 30,000) is also excluded: not only are there no a priori reasons why the glow should then have spread after CN treatment, but, in fact, the glow was always within the cell boundaries when