show that single copies of the genes encoding the two PA's that have been characterized in mammalian cells are located on human chromosomes 8 and 10. This conclusion is inconsistent with the previous assignment by Kucherlapati *et al.* of a PA gene, probably the u-PA gene, to chromosome 6 (*15*). DNA from hybrids containing chromosome 6 but not 8 (TSL-2, DUM-13, JSR-14, and JWR-22H) did not show any hybridization with the t-PA probe. Similarly, DNA from hybrids retaining chromosome 6 but not chromosome 10 (JSR-14) did not hybridize with the u-PA probe, and, further, DNA from hybrid JSR-14 (which contains chromosome 6, but neither chromosome 8 or 10) also did not hybridize with either probe. Kucherlapati *et al.* (*15*) relied on the expression of a PA gene in hybrid cells as the means of determining the presence of the gene. Hybridization of structural gene sequences to specific cDNA probes is a more direct and sensitive technique that does not depend on the expression of the gene to be mapped in hybrid cells and hence avoids the uncertainty that a regulatory gene is being identified in hybrids. Blasi and his colleagues have independently localized the u-PA gene to human chromosome 10 (*16*).

Tissue-PA and u-PA belong to the family of serine proteases, a group of enzymes related by structure and function, that may have evolved from a common ancestral gene (*17*). The genes for trypsin; chymotrypsin B; elastase; coagulation factors IX, X, and XII; plasminogen; complement component C3; and haptoglobin are located on human chromosomes 7, 16, 12, X, 13, 6, 6, 19, and 16, respectively (*18*). Although haptoglobin is not a serine protease, it is thought to have evolved from the same ancestral gene as the serine proteases (*19*). The presence of serine protease genes on different chromosomes implies that the duplication and the divergence from the common ancestral gene occurred early in evolutionary history.

In view of the association of PA's with tumor formation and metastasis, it may be significant that two oncogenes, c-*mos* and c-*myc*, have been mapped to human chromosome 8 (*20*). A gain of chromosome 8 is the most common change observed in chronic myelogenous leukemia and acute nonlymphocytic leukemia, and translocations and rearrangements of chromosomes 8 have also been linked to Burkitt lymphoma and acute myelogenous leukemia (*21*). It is therefore of interest that circulating cells that secrete t-PA appear to be characteristic of a particular subpopulation of adult human leukemia patients who are refractory to conventional antileukemic chemotherapy (*22*). In addition, fragile sites have been found on chromosomes 8 and 10; fragile sites have been associated with breakpoints in chromosomal translocations (*23*). However, it is not known if the t-PA or the u-PA gene locus is translocated or rearranged in any human cancer.

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Phytochelatins: The Principal Heavy-Metal Complexing Peptides of Higher Plants

Abstract. *A set of novel heavy-metal complexing peptides was isolated from plant cell suspension cultures; the structure of the peptides was established as (γ-glutamic acid–cysteine)*n*-glycine* (n = *3 to 7*). *These peptides appear upon induction of plant cells with heavy metals and represent the principal metal-binding activities in the cells. The name phytochelatin is proposed for this new class of natural products.*

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Heavy metals in vertebrates and fungi are detoxified by the metallothioneins, which are sulfur-rich proteins 6.5 kilodaltons (kD) in size (*1*). Mammalian metallothioneins in general consist of a single polypeptide chain with 61 amino-acid residues (*1*). Metal chelating compounds have also been proposed for higher plants (*2*), and a metallothionein has been reported in a vascular plant (*3*). Further characterization of this protein revealed that it bears a close resemblance to vertebrate metallothioneins (*4*). Recently, several 10-kD metal-binding metallothionein-like proteins from differentiated tissues and cell cultures of higher plants have been reported (*5–9*).

Suspension cultures of *Rauvolfia serpentina* were grown under sterile conditions for 6 days in the medium described (*10*). The medium was then augmented with 200 μM $CdSO_4$, and the cell suspension was cultured for another 4 days. Cells were harvested by suction filtration, washed extensively with deionized water, dried by suction, and frozen in liquid nitrogen. All extraction and isolation procedures were carried out at 0° to 4°C under nitrogen.

A typical separation of a crude cell-free extract of Cd^{2+}-treated and untreated (control) cells on Sephadex G-50 is shown in Fig. 1. The extract from Cd^{2+}-treated cells (Fig. 1B) was resolved into high molecular weight material containing approximately 3 percent of the total

Cd^{2+}, a major Cd^{2+}-free peak of low molecular weight, and an intermediate peak of material containing more than 90 percent of the total Cd^{2+} as well as –SH groups. This second Cd^{2+}-containing peak was not detected in extracts from untreated controls or in crude extracts of untreated cells after addition of $CdSO_4$ (Fig. 1A).

The metal-induced material was isolated on a preparative scale by DEAE-Biogel and ammonium-sulfate fractionation, ultrafiltration (Amicon YM2), Sephadex G-50 chromatography, and lyophilization. Typically, 160 mg of Cd^{2+}-containing material was isolated from 1 kg (fresh weight) of cells. Subsequent high-performance liquid chromatography (HPLC; Nucleosil C-18) at *p*H 2.7 (0.05 percent phosphoric acid in 0 to 20 percent acetonitril–H_2O) separated this material into five metal-free peaks absorbing at 220 nm. The major component, representing approximately 60 percent of the material, was isolated preparatively and analyzed further. Molecular weight determination by size-exclusion HPLC (*11*) in 6*M* guanidinium hydrochloride gave a relative molecular weight of 1040 for the denatured metal-free form. Analysis under nondenaturing conditions of a Cd^{2+}-reconstituted sample yielded a relative molecular weight of 3500 for the metal-containing compound. These data suggest a trimeric structure for the native Cd^{2+}-containing compound.

The ultraviolet absorbance spectrum of the compound below 300 nm closely resembles that for Cd^{2+}-containing metallothioneins (*12*). Upon treatment with HCl to *p*H 0.4, the absorbance leveled off because of the destruction of Cd^{2+}-thiolate coordination (Fig. 2A). Circular dichroism (CD) analysis (Fig. 2B) of this material yielded Cotton extrema similar to those in vertebrate Cd^{2+}-metallothioneins (*1*), presenting specific evidence for Cd^{2+}-thiolate structural elements.

Analysis of the amino acid composition of the purified, Cd^{2+}-free compound showed only L-cysteine, L-glutamic acid, and glycine in a ratio of 4:4:1. Attempts to sequence the peptide through direct Edman degradation were not successful. Hydrazinolysis of the peptide gave free glycine. Dinitrophenylated peptide yielded 1 mole of dinitrophenylated glutamic acid per mole of peptide upon acid hydrolysis. Treatment of the *S*-benzylated peptide with γ-glutamyl transferase (*13*) yielded 1 mole of glutamic acid per mole of peptide. The residual peptide was subjected to a modified Edman degradation (*14*) and yielded *S*-benzylated cysteine. By cyclic repetition of the γ-glutamyl transferase and the Edman degradation treatment, the complete sequence of the peptide could be elucidated. Furthermore, the dinitrophenylated and performic acid–oxidized peptide was partially hydrolyzed (1*N* HCl; 18 minutes at 95°C), and the peptides thus generated were separated by two-dimensional thin-layer chromatography.

The amino acid sequence of these peptides as determined by the above procedure was in full accord with the proposed structure. The structure of the metal-free monomeric peptide therefore is $(NH_3)^+$-γ-Glu-Cys-γ-Glu-Cys-γ-Glu-Cys-γ-Glu-Cys-Gly-COO^-. Elementary analysis of the purified peptide gave the following percentages for the atomic constituents: C, 40.41; H, 5.14; O, 29.06; N, 12.63; and S, 12.75. The formula $C_{34}H_{53}O_{18}N_9S_4$ requires the following percentages: C, 40.67; H, 5.28; O, 28.71; N, 12.56; and S, 12.78. Analysis of the native, Cd^{2+}-containing mixture of phytochelatins purified up to the HPLC step yielded a Cd^{2+}:cysteine ratio of about 1:2, while in vertebrate metallothioneins it is 1 : 3. No metal other than Cd^{2+} was detected by atomic absorption spectrophotometry. The primary structure of the nonapeptide was confirmed by chemical synthesis. Sequence analysis of the other four peptides isolated by preparative HPLC as minor components yielded the following structure: $(\gamma\text{-Glu-Cys})_n$Gly ($n$ = 3, 5, 6, and 7).

The synthesis of phytochelatins is induced not only by Cd^{2+} but also by other heavy-metal ions such as Cu^{2+}, Hg^{2+}, Pb^{2+}, and Zn^{2+}. For each of the ions, the metal phytochelatins were isolated from *R. serpentina* cell cultures. Cell extracts not exposed to metals did not yield any phytochelatins within the detection limit. Cell cultures of *Rosa canina*, *Silene cucubalus* (Dicotyledoneae), and *Dioscorea composita* (Monocotyledoneae) synthesized large amounts of the peptides when exposed to Cd^{2+}. In each case, the peptides were isolated and analyzed for their

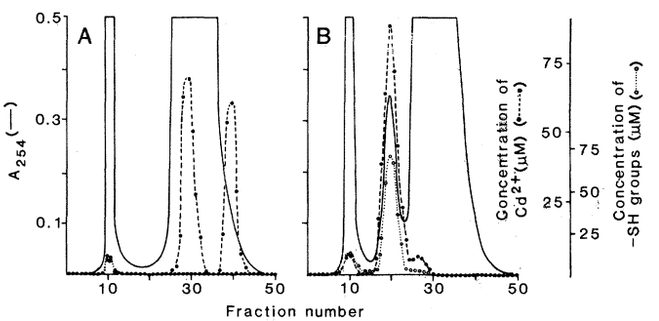

Fig. 1. Elution profiles of crude extracts from *Rauvolfia serpentina* suspension cultures grown in the absence (*A*) and presence (B) of $CdSO_4$. Frozen cells were thawed in one half-volume of 10 m*M* tris-HCl (*p*H 8.6) and centrifuged at 10,000*g* for 30 minutes. The supernatant was concentrated by lyophilization, and a sample containing 20 mg of protein was applied to a Sephadex G-50 column (2.5 by 51 cm) equilibrated with 10 m*M* tris-HCl (*p*H 8.0) and 0.1*M* NaCl. Proteins were eluted with the same buffer (flow rate, 73 ml $hour^{-1}$; fraction volumes, 7.3 ml). Concentration of Cd^{2+} was measured by atomic absorption spectrophotometry (Perkin-Elmer, flame mode), and SH content was measured with Ellman's reagent (*15*). In (A), no Cd^{2+} could be detected throughout the gradient. When $CdSO_4$ (380 μg per 20 mg of protein) was added to these extracts before Sephadex G-50 chromatography, no Cd^{2+} could be detected at the phytochelatin position. The two major peaks at lower retention times represent, respectively, free Cd^{2+} and Cd^{2+} bound to unidentified low molecular weight (<500) material.

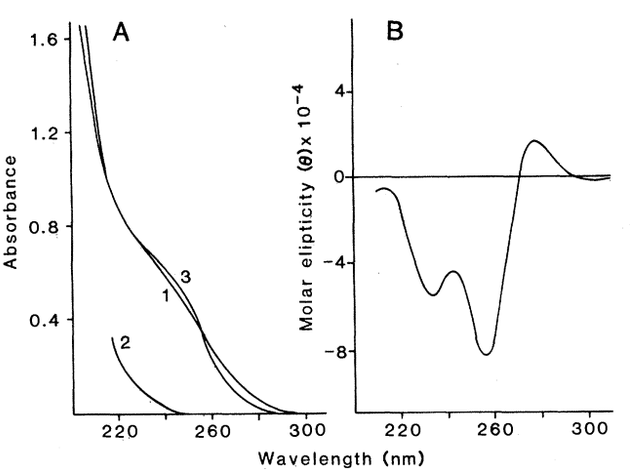

Fig. 2. Ultraviolet absorbance and CD spectra of phytochelatin. (A) Ultraviolet spectrum of the purified, Cd^{2+}-containing peptide in 5 m*M* tris-HCl (*p*H 7.8) (peak 1) after acidification with concentrated HCl to *p*H 0.4 (peak 2) and after readjusting the *p*H to 7.8 with 5*M* NaOH under a stream of nitrogen (peak 3). (B) CD spectrum of purified peptide in 50 m*M* sodium phosphate buffer (*p*H 7.0). Molar ellipticity is given in units of degrees per square centimeter per decimole.

amino acid composition, amino acid ratios, and CD spectra. By these criteria, all phytochelatins were indistinguishable from those isolated from *R. serpentina*.

The induction of the peptides by Cd^{2+} was also observed in cell cultures of *Anethum graveolens*, *Berberis stolonifera*, *Catharanthus roseus*, *Fumaria parviflora*, *Galium mollugo*, *Malva sylvestris*, *Rhazya stricta*, *Solanum marginatum*, and *Thalictrum dipterocarpum*. In all cases, more than 90 percent of the Cd^{2+} taken up by the cells was complexed to the phytochelatin peptides. The traces of Cd^{2+} (3 percent) that were excluded from Sephadex G-50 (Fig. 1B) were associated with proteinaceous material with a relative molecular weight of more than 30,000. There was no indication of the formation of a 10-kD metallothionein as reported previously (*5–9*). However, in our system the metal ions were supplied at a 200 μM concentration, which is about 10 times higher than that used by others (*5–9*).

The phytochelatins may be viewed as linear polymers of the γ-Glu-Cys portion of glutathione and, indeed, may be formed from glutathione itself. Because of the repetitive γ-glutamic acid bonds, they cannot be regarded as primary gene products. Phytochelatins are apparently the simplest (composed of only three different amino acids) natural compounds so far reported that may be engaged in the detoxification and homeostasis of heavy metals through metal-thiolate formation. They are completely different in structure from the metallothioneins reported earlier but may serve the same purpose of binding excess heavy metals through mercaptide complexes.

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Wounding and Its Role in RSV-Mediated Tumor Formation

Abstract. *Tumors induced in chickens by Rous sarcoma virus remain localized at the site of injection even though the animals become viremic. Tumors have now been shown to be inducible at other sites if a wound is inflicted or if the tissue is injured by administration of tumor promoters. These findings indicate that local wounding plays a role in the spread of tumorigenicity of Rous sarcoma virus.*

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In most studies of tumor formation mediated by Rous sarcoma virus (RSV), the virus is administered by subcutaneous or intramuscular injection, a process that creates some local wounding (*1*). Viral infection of newly hatched chicks results in the rapid growth of a localized sarcoma that becomes palpable within 1 to 2 weeks, in the production of circulating progeny virus, and in most cases, in the death of the host within a month (*2*). Tumors usually form only at the site of inoculation (*3*), whereas nonmalignant hemorrhagic lesions are often found in tissues throughout the animal (*1*). Occasionally, distal tumors have been reported in young chicks, but they appear with a much longer latency and only in addition to the rapidly forming local tumor (*4, 5*). If circulating virus is present and if, as has been proposed, RSV infection and concomitant *src* gene expression are sufficient for neoplastic transformation and sarcoma development in chickens (*6*), tumors should form elsewhere as well.

To determine whether progeny virus was indeed present throughout the animal, we assayed several types of tissues obtained from tumor-bearing chickens for the presence of focus-forming units (ffu). Tissues were minced in buffer, serially diluted, and tested for their ability to transform cultured chicken embryo fibroblasts (CEF) in a focus assay (*7*). Progeny virus was present in all tissues assayed (Table 1). Although this does not constitute proof that the virus was present intracellularly, it does indicate that it was being circulated. Despite this circulating infectious virus, tumors formed preferentially at the site of inoculation; it was thus reasonable to suspect that either wounding associated with inoculation or the subsequent healing plays a part in the formation of tumors.

To test this hypothesis, we inoculated 10-day-old chicks intramuscularly in the right wing with 5×10^6 ffu of the Schmidt-Ruppin-D strain of RSV in a volume of 0.1 ml. The opposite wing was pierced with a small stainless steel clip that remained in place for the duration of the experiment (Fig. 1A). As expected, palpable tumors formed at the site of injection in all animals after 8 or 9 days. When a clip was inserted at the time of injection, tumors also formed at the site of the clip, with the same frequency as those inserted after injection, but with a 20 percent longer latency period (Fig. 1C). Tumors induced by injection were indistinguishable from those induced by wounding, as judged from histological examination of the tissue sections (Fig. 1B). The timing of the inflicted wound affected the latency of the resulting tumors. The longer the clip insertion was delayed after virus injection (up to 2 weeks), the shorter the latency in formation of the wound tumor (however, it was never less than 2 days) (Fig. 2). After 2 weeks, the animals either died or were killed. We speculate that the

Table 1. Seven-day-old chickens were given injections of SRD-RSV (5×10^6 ffu), and a clip was inserted into the opposite wing. Two weeks later, the animals were killed, and the tissues of interest were prepared for analysis. Focus-forming activity, expressed as focus-forming units per milligram of protein, and *src* kinase activity, expressed as the multiple of increase over corresponding normal tissue, were determined as described (*7*). The values are means ± standard error of the mean for three separate experiments.

Tissue	Infectious virus (ffu/mg)	Kinase activity (multiple increase)
Tumor at injection site	$1 \times 10^5 \pm 8.5$	25.4 ± 2.7
Spleen	$4 \times 10^4 \pm 3.1$	6.0 ± 2.9
Breast	$1 \times 10^3 \pm 0.4$	1.0 ± 0.1
Tumor at clip site	$2 \times 10^6 \pm 1.2$	14.3 ± 3.0