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Involvement of a Vitronectin-Like Protein in Attachment of *Agrobacterium tumefaciens* to Carrot Suspension Culture Cells

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Infections of dicotyledonous plants by *Agrobacterium tumefaciens* result in the formation of crown gall tumors. Attachment of the bacteria to plant host cells is required for tumor formation. Human vitronectin and antivitronectin antibodies both inhibited the binding of *A. tumefaciens* to carrot cells. Wild-type bacteria are able to bind radioactive vitronectin; nonattaching mutants showed a reduction in the ability to bind vitronectin. The binding of biotype 1 *A. tumefaciens* to carrot cells or to radioactive vitronectin was not affected by high ionic strength. Detergent extraction of carrot cells removed the receptor to which the bacteria bind. The extract was found to contain a vitronectin-like protein. These results suggest that *A. tumefaciens* utilizes a vitronectin-like protein on the plant cell surface as the receptor for its initial attachment to host cells.

Infections of wound sites in dicotyledonous plants by the gram-negative bacterium *Agrobacterium tumefaciens* result in the formation of crown gall tumors. The tumors result from the transfer of a piece of DNA (the T-DNA) from the bacterium to the plant host cell. This DNA becomes integrated into the plant DNA, where it codes for the uncontrolled synthesis of plant growth hormones, thus resulting in the formation of a tumor (26). One of the early steps in bacterial pathogenesis is the attachment of the bacteria to plant host cells (13). This attachment is a two-step process. In the first step, the bacteria bind loosely to the plant surface. In the second step, substances released from the plant stimulate the bacteria to elaborate cellulose fibrils. These cellulose fibrils cause the bacteria to bind very tightly to the plant cell surface (15). The fibrils also entrap additional bacteria, resulting in the formation of bacterial aggregates. The majority of the bacteria in these aggregates are bound only indirectly to the plant cell via the network of cellulose fibrils (18).

Bacterial mutants which are unable to carry out the first step of loose binding to host cells are avirulent (5, 16, 31). Bacterial outer membrane proteins have been implicated in this initial attachment to the host (16). However, the identity of the host cell receptor to which the bacteria bind is unknown. The receptor can be removed from the surface of carrot cells by treatment with trypsin or other proteases or by extraction with dilute detergent (9, 23). The host range of biotype 1 strains of *A. tumefaciens* is very broad, suggesting that the receptor must be found on the cell surface of many species of plants (4). These observations suggest that the receptor may be a cell surface protein which is highly conserved in evolution.

Vitronectin (S protein) is a serum spreading factor found in animals (32). It is an important constituent of the extracellular matrix and is localized at focal adhesions or contact sites in cultured mammalian cells (1). The properties of vitronectin and its role in adhesion and in the stabilization of the cytoskeleton have been discussed in several recent reviews, for example, that by Tomasini and Mosher (32). Vitronectin has been reported to be bound by certain strains of several species of bacteria, including *Staphylococcus aureus*, several groups of streptococci, *Escherichia coli*, and *Actinomyces viscosus* (3, 7, 24). It is thought that vitronectin binding may play a role in host colonization and pathogenesis of these organisms (6). Recently, a vitronectin-like protein and its associated integrin (29) from several species of plants, including tomato, soybean, broad bean, and lily (27), and tobacco tissue culture cells (35, 36), have been described. *A. tumefaciens* is capable of inducing tumors on all of these plants (4, 12). The bacteria bind to cells from all susceptible species which have been examined.

Sources and growth of cultures. Bacteria were grown and viable counts were determined as previously described (19). The strains of bacteria used and their characteristics are listed in Table 1. All of these strains except Att-339 have been described previously. Strain Att-339 was constructed by marker exchange into the wild-type strain A6 of the cloned transposon insertion from the C58 attachment mutant Att-C43 (16). Att-339 was unable to attach to carrot suspension culture cells and was avirulent on *Bryophyllum daigremontiana* leaves. It produced roughly the same amount of cellulose as the parent strain as judged by fluorescence on medium containing 0.02% cellufluor.

Carrot suspension culture cells were grown in Murashige and Skoog medium (22), with weekly transfers as described previously (18).

Measurement of bacterial attachment to plant cells. Attachment of bacteria to plant cells was measured by the procedure of Matthysse et al. (18). Freshly diluted carrot cells were used at 2×10^5 to 75×10^5 cells per ml in Murashige and Skoog medium; suspensions of bacteria were added to the carrot cultures to a final concentration of approximately 3×10^3 bacteria per ml. The number of bacteria attached to the plant cells was determined after various incubation times by filtering an aliquot of the bacterial carrot cell suspension mixture through Miracloth (Calbiochem), which allowed the passage of free bacteria but not of the carrot cells; the number of free bacteria and the number bound to the carrot cells were determined by viable cell counts after vortexing of free bacteria and grinding of bound bacteria and carrot cells in a Waring blender. These procedures were observed with

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TABLE 1. Bacterial strains used in this study

Bacterial strain	Relevant characteristic(s)	Source or reference
A. tumefaciens A6		
Parent strain	Wild type	A. Matthysse
Mutants		
Cel-12	A6::Tn*5*, cellulose-minus, attaches, virulent	14
Ivr-225	A6::Tn*5*, modified lipopolysaccharide, attaches, avirulent	20
Att-339	A6::Tn*5 att*, nonattaching, avirulent	This paper
PscA	A6::Tn*5*, exopolysaccharide mutant, *pscA*, nonattaching, avirulent	31
A. tumefaciens C58		
Parent strain	Wild type	A. Matthysse
Mutants		
Att-C43	C58::Tn*5 att*, nonattaching, avirulent	16
Att-C69	C58::Tn*5 att*, nonattaching, avirulent	16
A1045	C58::Tn*5*, β-1,2-D-glucan-minus *chvB*, nonattaching, avirulent	5
R. meliloti 1021	Wild type	S. Long

TABLE 2. Effect of various substances on attachment of *A. tumefaciens* Cel-12 to carrot suspension culture cells

Addition to medium	% of control attachment[a]
None	100 (control)
Antifibronectin antibody, 1/5,000 dilution	101 ± 24
Antivitronectin antibody	
1/5,000 dilution	13 ± 13
1/10,000 dilution	10 ± 10
1/50,000 dilution	100 ± 20
Vitronectin, 20 ng/ml	50 ± 15

[a] Attachment data (± standard deviation) for 120-min incubations.

the light microscope to result in the formation of a single-cell suspension of the bacteria. The effect of various additions to the medium on bacterial attachment was measured. Polyclonal antihuman vitronectin or antihuman fibronectin antibodies purchased from Telios Pharmaceuticals were added to the carrot cells 45 min before the addition of the bacteria. Vitronectin (Telios Pharmaceuticals) was added to the carrot cells immediately before the addition of the bacteria. Fibronectin is an animal protein closely related to vitronectin (32). However, unlike vitronectin, fibronectin is not known to occur in unstressed higher plant cells. Therefore, we used antifibronectin antibodies as a control for the specificity of the interactions with antivitronectin antibodies.

Labeling of vitronectin and measurement of its binding to bacteria. Human vitronectin (Telios Pharmaceuticals) was labeled with ^{125}I (Amersham) by using Iodobeads (Pierce) and the protocol provided by the manufacturer. The specific activity obtained was 10^6 cpm/μg. The ability of various strains of bacteria to bind the radioactive vitronectin was measured. Fresh overnight cultures of bacteria were grown in Luria broth, and the cells were collected by centrifugation for 5 min in an Eppendorf centrifuge. The bacteria were resuspended in a siliconized Eppendorf tube in Murashige and Skoog medium, unless otherwise indicated, to a final concentration between 3×10^8 and 7×10^9 bacteria per ml. Radioactive vitronectin was added to a final concentration of 4 ng/ml. The bacteria and vitronectin were incubated together for 20 min on a shaker at 150 rpm at room temperature. The bacteria were then collected by centrifugation in an Eppendorf centrifuge, washed once with water, resuspended in water, and collected on a Millipore GS 0.2-μm-pore-size filter. The filter was washed four times with water and dried, and the radioactivity was counted in a liquid scintillation counter. All manipulations involving vitronectin were carried out with siliconized glass and plastic.

Detection of a protein which cross-reacts with antivitronectin antibodies from carrot cells by immunoblots. Surface component proteins were extracted from carrot suspension culture cells by exposure to 0.1% Triton X-100 according to the method of Gurlitz and Matthysse (10). This protocol has been shown to remove the binding site for *A. tumefaciens* from carrot cells (10). After the extract was concentrated by using Aquacide I-A (Calbiochem), it was dialyzed, resuspended in sample buffer, and clarified by centrifugation. The total protein concentration was determined by the amido black procedure (28). Purified human vitronectin (Vn) protein (Telios Pharmaceuticals) was dissolved at a concentration of 1 mg/ml in phosphate-buffered saline (PBS; per liter, 80 g of NaCl, 2 g of KCl, 2 g of KH_2PO_4, 11.5 g of Na_2HPO_4) and stored at −20°C.

The protein extracts from the carrot cells and purified human vitronectin (Vn) were electrophoresed in a 12.5% polyacrylamide vertical gel and blotted onto Hybond N (Amersham). Papers carrying blotted proteins were blocked overnight in 8% (wt/vol) nonfat powdered milk in PBS at 4°C and then incubated for 1 to 2 h at room temperature with primary antibody (rabbit antihuman vitronectin; Telios Pharmaceuticals) at a 1:500 dilution in PBS containing 0.1% Tween (Sigma Chemical Company) and 0.2% bovine serum albumin (BSA) (Sigma Chemical Company) (PBSTB). After three washes in PBSTB, the immunoblots were incubated with secondary antibody (goat anti-rabbit immunoglobulin G–alkaline phosphatase conjugate; Sigma Chemical Company) for 1 h at a dilution of 1:1,000 with PBSTB. After three additional washes, the blots were incubated with color development solution (ProtoBlot System; Promega). Nonimmune rabbit serum (gift of Alan Jones, University of North Carolina, Chapel Hill) and the secondary antibody alone were used as controls for detecting nonspecific antibody binding. A sodium metaperiodate oxidation of the Western blots (immunoblots) was carried out according to the procedure of Woodward et al. (34) to determine whether the polyclonal antivitronectin antibodies were detecting carbohydrate or noncarbohydrate determinants.

Effect of vitronectin and antivitronectin antibodies on bacterial attachment. To examine only the first step in bacterial attachment, all measurements of attachment were done by using a mutant, Cel-12, which is unable to make cellulose fibrils and thus does not form bacterial aggregates but which binds to the plant cell and is virulent (14). Attachment of Cel-12 to carrot cells was unaffected by the addition of antifibronectin antibodies to the culture medium. However, attachment was inhibited by the addition of antivitronectin

TABLE 3. Binding of radioactive vitronectin by and attachment to carrot cells of various bacterial strains

Bacterial strain	Binding (% of control)	
	Vitronectin[a]	Carrot cells[b]
R. meliloti 1021[c]	2 ± 2	10 ± 10
A. tumefaciens A6		
Parent strain	100 (control)	100 (control)
Mutants		
Cel-12	91 ± 26	60 ± 15
Ivr-225	98 ± 17	100 ± 25
Att-339	15 ± 15	10 ± 10
PscA	24 ± 15	20 ± 20
A. tumefaciens C58		
Parent strain	100 (control)	100 (control)
Mutants		
Att-C43	30 ± 15	7 ± 7
Att-C69	35 ± 30	7 ± 7
A1045	4 ± 4	28 ± 28

[a] The control bound between 400 and 800 pg of vitronectin per 10^9 bacteria.
[b] Binding data for carrot cells are given for 20-min incubation in Murashige and Skoog medium. The percent of the bacterial inocula bound for the controls was 35% of the A6 strain and 20% of the C58 strain.
[c] The control used in calculating the relative binding of *R. meliloti* was the A6 strain of *A. tumefaciens*.

antibodies. The addition of vitronectin to the medium also inhibited the attachment of the bacteria (Table 2).

Binding of vitronectin to bacteria. Since the addition of antivitronectin antibodies or vitronectin inhibited the binding of *A. tumefaciens* to carrot cells, it was of interest to determine whether the bacteria themselves could bind vitronectin. Wild-type A6 bacteria were able to bind radioactive vitronectin (Table 3). At the concentrations tested, the amount of vitronectin bound was proportional to the number of bacteria added to the incubation mixture for the range from 3×10^8 to 7×10^9 bacteria per ml and 4 ng of vitronectin per ml. The addition of a 100-fold excess of cold vitronectin prior to the addition of the radioactive vitronectin reduced the binding of the radioactive vitronectin by more than 95%. The addition of a 1,000-fold excess of BSA prior to the addition of the radioactive vitronectin had no effect on bacterial binding of vitronectin.

The time course of the binding of vitronectin to bacteria was examined by using bacterial strain Cel-12, which is unable to synthesize cellulose fibrils and thus does not clump in plant tissue culture medium. The bacteria had already reached the maximum level of vitronectin binding after 10 min of incubation. The binding was constant for the next 100 min, which was the longest time of incubation examined. Shorter times of incubation were not examined since the collection and washing of the bacteria took a minimum of 15 min.

Effect of medium ionic strength on vitronectin binding by *A. tumefaciens*. The binding of biotype 1 strains of *A. tumefaciens* to carrot cells is unaffected by changes in the ionic strength of the medium (30). Therefore, we examined the effect of ionic strength of the medium on the ability of strain A6, which is a biotype 1 strain, to bind radioactive vitronectin. The bacteria bound about 400 pg of vitronectin per 10^9 bacteria whether the binding was carried out in 4% sucrose, Murashige and Skoog medium, or Murashige and Skoog medium with 0.25 M NaCl. Thus, the ionic strength of the medium did not effect the ability of the bacteria to bind vitronectin.

Attachment to carrot cells and binding of vitronectin by various bacterial strains and mutants. Wild-type bacteria of both the A6 and C58 strains bound to carrot cells during a 20-min incubation in Murashige and Skoog medium (Table 3). The binding of various bacterial mutants to carrot cells was examined. The Ivr-225 mutant was unaltered in binding to carrot cells. The cellulose-minus mutant, Cel-12, was slightly reduced in binding, presumably because of the lack of the formation of bacterial aggregates on the carrot cell surface (14). Mutants which have previously been characterized as nonattaching, including PscA, Att-C43, Att-C69, and A1045, showed no significant attachment to carrot cells (2, 5, 16, 31). The Att-339 mutant also failed to bind to carrot cells. *Rhizobium meliloti*, which is closely related to *A. tumefaciens*, also showed no significant attachment to carrot cells (17).

The ability of these various bacterial strains and mutants to bind radioactive vitronectin was examined. The virulent wild-type *A. tumefaciens* A6 and C58 bound ^{125}I-labeled vitronectin. Similarly, a cellulose-minus mutant (Cel-12) and an avirulent lipopolysaccharide mutant (Ivr-225), both of which still bind to carrot cells, also bound the radioactive vitronectin. Three types of nonattaching mutants have been described for *A. tumefaciens* (5, 16, 31). The avirulent nonattaching mutants Att-339, Att-C43, and Att-C69 that all contain transposon insertions in *att* showed a marked reduction in vitronectin binding. The avirulent nonattaching mutants A1045 and PscA, which are mutant at the *chvB* and *pscA* (*exoC*) loci, respectively, also were reduced in their ability to bind vitronectin. *R. meliloti* 1021 showed almost no binding to the vitronectin protein; these bacteria also showed no significant binding to carrot suspension culture cells. The ability of the various bacterial mutants to bind radioactive vitronectin correlates with their ability to bind to carrot cells (Table 3).

Detection of a protein which cross-reacts with antivitronectin antibodies in carrot cell extracts. Extraction of carrot suspension culture cells with 0.1% Triton X-100 has been previously shown to remove the bacterial binding site from the carrot cells without affecting their viability (9). When the proteins from these extracts were separated by polyacrylamide gel electrophoresis and subjected to immunoblot analysis by using polyclonal antibodies to human vitronectin (Vn), two polypeptides with sizes of approximately 65 and 55 kDa reacted with the antibody (Fig. 1). The specificity of the immunological cross-reactivity of the human vitronectin antibodies was evaluated by using nonimmune and secondary antibodies. The nonimmune serum and the secondary antibody alone (Fig. 1) failed to recognize the 55-kDa protein; however, a very small amount of cross-reactive material was observed at 65 kDa, suggesting that the 55-kDa molecule and possibly the 65-kDa molecule are vitronectin-like proteins. The polyclonal antibodies still recognized the vitronectin-like molecules after a metaperiodate oxidation of the immunoblot (data not shown), suggesting that the cross-reacting antibodies were detecting polypeptide rather than carbohydrate determinants.

The results presented above suggest that *A. tumefaciens* binds to a vitronectin-like protein on the surface of plant cells. The binding of the bacteria to carrot cells was inhibited by antibodies to vitronectin but not by antibodies to fibronectin. In addition, binding was also inhibited by added vitronectin (Table 2). *A. tumefaciens* was capable of binding radioactive vitronectin (Table 3). *R. meliloti*, which is closely related to *A. tumefaciens* but which failed to bind to carrot cells, also failed to bind vitronectin. Bacterial mutants

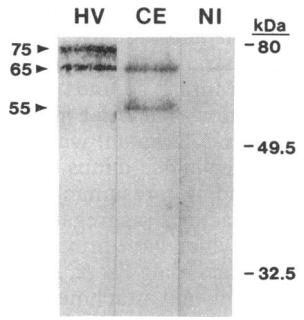

FIG. 1. Immunoblot detection of vitronectin and vitronectin-like proteins. Lanes: HV, human vitronectin with two bands at 75 and 65 kDa (indicated on the left) after reaction with polyclonal antihuman vitronectin antiserum; CE, detergent-extracted surface components of carrot suspension culture cells showed two bands at 65 and 55 kDa by using the same polyclonal antihuman vitronectin serum; NI, the same carrot extract after reaction with a nonimmune antibody control detected only a small amount of cross-reactive material at 65 kDa. Primary antibody omission (data not shown) showed no cross-reactive material in the carrot cell extracts. The positions of molecular weight markers are shown on the right.

which are unable to bind to plant cells also showed reduced binding of vitronectin (Att-339, Att-C43, Att-C69, A1045, and PscA). The strains studied included representatives of each of the three types of nonattaching mutants which have been described for *A. tumefaciens*. Mutations in the *chvB* gene are the most studied of these. *chvB* encodes an enzyme for the synthesis of β-1,2-D-glucan. Mutations in this gene have a pleiotropic effect: the bacteria show reduced motility, increased production of extracellular polysaccharide, failure to bind to plant cells, and avirulence as well as absence of the β-1,2-D-glucan (5, 25). Mutations in *pscA* (*exoC*) result in a general defect in the production of extracellular polysaccharides. These mutants also fail to bind to plant cells and are avirulent (2, 31). Mutations in *att* genes lack one or more of three minor outer membrane proteins. They have no known alterations in polysaccharides. *att* mutants also fail to bind to plant cells and are avirulent (16). The only characteristics shared in common by *chvB*, *pscA*, and *att* mutants are their failure to bind to plant cells, avirulence, and reduced ability to bind vitronectin.

In contrast, bacterial mutants altered in surface properties such as cellulose synthesis (Cel-12) or lipopolysaccharide structure (Ivr-225) which do not affect the ability of the bacteria to bind to plant cells were able to bind vitronectin (Table 3). The binding of biotype 1 *A. tumefaciens* to carrot cells is insensitive to alterations in the ionic strength of the medium (30). The binding of vitronectin by *A. tumefaciens* A6, a biotype 1 strain, was also unaffected by alterations in the ionic strength of the medium. Thus, the binding of the bacteria to carrot cells parallels the binding of bacteria to vitronectin in a manner consistent with a role for vitronectin in the binding of the bacteria to plant cells.

The same polyclonal antivitronectin antibodies that inhibited bacterial binding to carrot cells also recognized a protein in detergent extracts of the carrot cells. The extraction procedure used (0.1% Triton X-100) removes the receptors to which the bacteria bind from the carrot cells yet does not affect their viability (9). These results suggest that antihuman vitronectin serum inhibits the attachment process by binding to an exposed cross-reacting protein on the surface of the carrot cells. The fact that vitronectin itself also inhibits bacterial binding makes it unlikely that the blocking of attachment could be due to steric hindrance by the antibody of another nearby protein on the carrot cell wall. The antigenic determinants recognized by the polyclonal antivitronectin antibodies are not known. Thus, it is not certain in what way the cross-reacting protein in the carrot extract resembles human vitronectin. These antihuman vitronectin antibodies react with vitronectin-like proteins from a wide variety of organisms, including the slime mold *Physarum polycephalum* (21), the brown alga *Fucus distichus* (33), and higher plants (27, 35, 36). It is possible that the similarity lies in a carbohydrate modification of the two proteins; however, the fact that treatment of the carrot extract with periodate did not reduce the reaction with the antivitronectin antibodies suggests that the carrot protein and human vitronectin share antigenic determinants which reside on the polypeptide chain (34).

Although bacterial binding may limit host range of *A. tumefaciens* in some plants (11), it does not appear that failure of bacterial attachment is responsible for all limitations of host range. In the case of *Zea mays*, on which the bacteria do not induce tumors, the bacteria are still capable of binding, although in very low numbers, to corn cells and tissues (8, 10). In attempting to correlate binding of *A. tumefaciens* with the presence of vitronectin on plant cells, it is important to remember that the bacteria are induced to make cellulose fibrils by the presence of plant cells. These fibrils may cause the bacteria to stick nonspecifically to cellulosic surfaces, including those of plant cells. The observations reported above are consistent with a role for plant vitronectin as the surface receptor to which *A. tumefaciens* binds since vitronectin is likely to be widely distributed in various plant species.

In conclusion, *A. tumefaciens* appears to bind to a vitronectin-like protein on the carrot cell surface. In this paper, we report the binding of human vitronectin to virulent wild-type *A. tumefaciens* strains and also the presence of a vitronectin-like protein in carrot suspension culture cells. We present evidence suggesting a functional role of this vitronectin-like protein in the adhesion of the bacteria to carrot suspension culture cells. We also speculate that the ability of the bacteria to attach to an exterior component of a structural and functional complex that may be linked via integrin to an actin cytoskeletal network could be an advantage for *A. tumefaciens*. The use of established actin tracks leading toward the interior of the cell might serve as a method of transport for T-DNA and associated proteins to the host cell nucleus.

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