RGD-dependent Linkage between Plant Cell Wall and Plasma Membrane: Consequences for Growth

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Abstract. Soybean (Glycine max [L.] Merr. cv. Mandarin) root cells (SB-1 cell line) grown in suspension culture containing Glycyl-Arginyl-Glycyl-Aspartyl-Seryl-Proline (GRGDSP) (0.25 mg/ml), a synthetic peptide containing the RGD sequence found in many extracellular matrix adhesive proteins, demonstrated (a) significantly enhanced growth rate, and (b) aberrant cell wall/plasma membrane interactions and organization. Substitution of the Asp (D) by a Glu (E) amino acid in the hexapeptide, or inversion of the RGD sequence to GDR, abolished the morphological and growth effects observed for GRGDSP in plant cells. Immunoblots, which were prepared from β-octylglucoside extracts of whole soybean cells and protoplasts, probed with polyclonal antibodies raised against human vitronectin receptor (hVNR) complex, demonstrated a single band with an apparent molecular mass of 2 kD. Chromatography of β-octylglucoside extracts of SB-1 cells on a Gly-Arg-Gly-Asp-Ser-Pro-Lys-sepharose affinity column demonstrated the retention of a single 70-72 kD polypeptide that reacted specifically with anti-hVNR antiserum. In contradistinction, no cross-reactivity was observed with antifibronectin receptor antiserum. Epifluorescence microscopy of whole soybean cells, after moderate treatment with pectinase, demonstrated punctate fluorescent patches at the cell membrane/wall boundary when probed with anti-hVNR and rhodamine-derivatized secondary antibodies. We propose that coordination and control of plant cell division and proper cell wall biosynthesis may be mediated by an RGD-dependent recognition system in which RGD binding protein(s) promote cell membrane–cell wall attachment.

The attachment of anchorage-dependent animal cells to extracellular matrix (ECM) is principally mediated by divalent cation dependent cellular recognition events between a class of plasma membrane receptors, collectively termed integrins (20, 44), and a family of ECM-localized adhesive glycoproteins, such as fibronectin and vitronectin (17, 21). A general feature of these interactions is that a number of integrins isolated from various normal and transformed cell lines can recognize the amino acid sequence Arg-Gly-Asp (RGD), which has been demonstrated to be a single or multiple unit in the polypeptide chains of fibronectin (37, 39), fibrinogen (15, 40), collagen (11), vitronectin (9, 41, 51), and von Willebrand factor (9, 15, 40). Synthetic peptides containing the RGD sequence can competitively inhibit the interaction between integrins and the RGD containing glycoproteins isolated from ECM (37-39). This specific recognition between cell and ECM has been implicated in cell migration (2, 7), transmembrane signaling events (44), differentiation (25, 44), and response to hormones and growth factors (22, 44). In transformed cells, integrins and their glycoprotein ligands may play a significant role in metastatic cell migration (19, 26, 44). It is clear that for tissue-forming animal cells, plasma membrane attachment to a collagen/proteoglycan composite is essential for cell organization and growth (16, 17, 20, 21, 44). This principle of membrane attachment to carbohydrate composite surfaces containing proteins and anionic polysaccharides would also appear to be relevant in discussions of the growth and proliferation of cell wall containing organisms such as bacteria and plants. In bacteria, membrane–cell wall interactions have been considered vital for the proper control and coordination of cell division (23). Indeed, morphologically distinct attachment sites between the cytoplasmic membrane and outer membrane/cell wall of gram-negative bacteria, termed zones of adhesion, have been suggested to be structures which are vital for cell biosynthesis and growth (5, 6). In plants, in a similar functional manner, must finely coordinate cell wall growth with cell division for proliferation (30, 32, 45). Enzymatic removal of plant cell wall results in a protoplast that apparently undergoes limited nuclear division but not cytokinesis (30, 32, 45). As with bacteria, plasmolysis of plant cells demonstrates points of continued attachment between plasma membrane sites and the cell wall. Examples of such plasmolyzed cells may be observed in the general botanical literature. Taken together, these observations may reflect a common
feature for normal cell growth and differentiation apparently crossing the evolutionary boundaries between prokaryotes and eucaryotes, namely a requirement for plasma membrane attachment to a custom synthesized carbohydrate composite secreted by the individual cell types. Recent reports demonstrating proteins containing potentially active RGD attachment sequences in bacteria (37, 38) and the possible importance of such sequences for parasite invasion (42), slime mold aggregation (13, 50), Drosophila embryonic development (25), and a report of a fibronectin-binding protein from Staphylococcus aureus (12) are, indeed, suggestive of a general membrane-wall-matrix recognition principle that may transcend species and kingdoms. In this context, the present communication examines the possibility that an RGD recognition system exists in higher plants that may play a role in the attachment of plasma membrane to cell wall. It is further suggested that this attachment may have significant consequences for cell growth. The data presented provide evidence that synthetic peptides containing the RGD sequence induce dramatic morphological and proliferative changes when introduced into growing suspension cultures of soybean (Glycine max [L.] Merr. cv. Mandarin) root cells (SB-1 cell line). These RGD-mediated proliferative changes appear to be mediated by a 70-72-kD polypeptide localized to the plant cell membrane/wall interface and isolated in β-octyl-glucoside extracts of soybean cells. Immunological cross-reactivity was observed between this 70-72-kD polypeptide and human vitronectin receptor (hVNR), providing a basis for the suggestion that RGD binding proteins exist in higher plants.

Materials and Methods

Reagents

GRGDS, GRGDSF, GRGESF, GRGDSPK, and SPGDRG peptides were custom synthesized by Dr. M. Pierschbacher (La Jolla Cancer Research Foundation, La Jolla, CA). Analysis for purity was performed by HPLC and mass spectral analysis at the Michigan State University National Institutes of Health Regional Mass Spectrometry Facility. To insure that these peptides were non-toxic for cell growth, an attachment inhibition assay was performed with BALB/3T3 fibroblasts, as previously described for Normal rat kidney cells, & immobilized fibronectin (37). Under conditions in which inhibition of attachment was observed, the cells were still viable as detected by exclusion of trypan blue. Anti-hVNR was a gift from Dr. E. Ruoslahti (La Jolla Cancer Research Foundation).

Cell Culture and Microscopic Analysis

Soybean (Glycine max [L.] Merr. cv. Mandarin) root cells (SB-1 cell line) were cultured for 20-72 h in IB5 medium (18, 29) in the absence or presence of the synthetic peptides (1 µg/ml-1 mg/ml of medium). Samples to be viewed under epifluorescent illumination were stained with rhodamine 123 (10 µg/ml for 10 min before viewing), a membrane potential fluorescent dye that accumulates in mitochondria (24). As previously demonstrated (24, 53), rhodamine 123 is a vital stain for plant and animal cells. Cell wall development in growing cells was examined with the dye Calcofluor, a fluorescent stain specific for polysaccharides with 1-4 linkages such as cellulose and chitin (27, 55). Samples of cells were stained with 0.1% Calcofluor in 0.4 M sorbitol for 1 min and then viewed with epifluorescent illumination (29). All phase and fluorescence analysis was performed on a epifluorescence microscope (E. Leitz, Inc., Rockleigh, NJ).

Proliferation Analysis

Cell proliferation was assayed in the following manner. An aliquot of cells in a constant volume (200 µl) was added to a Tared 1.5 ml tube (Eppendorf tubes made by Brinkmann Instruments, Inc., Westbury, NY) and the wet weight determined. Dry weights were obtained after dehydration of a constant volume of cells in tarred 1.5 ml tubes (Eppendorf tubes made by Brinkmann Instruments, Inc.). RNA and DNA content of cells in a constant volume was determined as described (8, 34). All time point weight measurements were in triplicate. Variability between samples for both wet weight and DNA determinations never exceeded 15%.

Isolation and Analysis of Arg-Gly-Asp Binding Protein from SB-1 Cells

Protoplasts were prepared from intact SB-1 cells as previously described (28, 29). 7.0 ml of packed protoplasts were solubilized with 10 ml of ethanol followed by addition of 20 ml of 200 mM octyl-β-glucoside (Calbiochem-Behring Corp., San Diego, CA) in PBS, pH 7.2, containing 1 M CaCl2, 1 M MgCl2, and 3 M PMSF. The ethanol was removed by evaporation under a nitrogen stream. The extraction was allowed to take place for 30 min at 4°C, after which insoluble cell debris was pelleted at 15,000 g. The supernatant was applied to a 1.5 ml column containing Gly-Arg-Gly-Asp-Ser-Pro-Lys-Sepharose (20 mg peptide/ml Sepharose) and allowed to bind overnight at 4°C. The column was washed with ~50-column volumes of PBS containing 50 mM octyl-β-glucoside and eluted with the same buffer containing 0.1 mg/ml Gly-Arg-Gly-Asp-Ser-Pro. The eluted material was concentrated ~10-fold by centrifugation through Centricon membranes (Amicon Corp., Danvers, MA) and examined by SDS-PAGE on 7.5% polyacrylamide gels stained with Coomassie blue or immunoblots probed with anti-hVNR antiserum or anti-hFNR antiserum (1:100 dilution for immunoblots). Transfer of SDS-PAGE gels to nitrocellulose was performed as described (52).

Immunofluorescence Localization

Soybean cells were grown in liquid cell suspension in IB5 medium (18, 29) for 48 h. Cell walls were permeabilized by the addition of 0.1 mg/ml pectinase for 15 min at room temperature (3). Plasmolyzed cells were prepared by transferring the pectinase treated cells from IB5 medium to modified Gamborg buffer containing sorbitol (0.4 M) and glucose (0.17 M). The presence of the 70-72-kD polypeptide was detected by indirect immunofluorescence, using rabbit anti-hVNR antibody and rhodamine conjugated goat anti-rabbit IgG as primary and secondary antibodies, respectively. Preimmune serum was used as primary antibody in control experiments.

Results

Morphological Analysis of Soybean Cells Grown in the Presence of RGD Peptides

Soybean (Glycine max [L.] Merr. cv. Mandarin) root cells (SB-1 cell line) were cultured for 20 h in IB5 medium in the absence or presence of either GRGDS, GRGES, or SPGDRG synthetic peptides to examine morphological effects. Samples stained with rhodamine 123, a membrane potential fluorescent dye and vital stain that accumulates in mitochondria (24, 53), were viewed using both phase contrast optics and epifluorescent illumination (Fig. 1). Stained mitochondria served to highlight the cytoplasmic compartment. Fig. 1, A and B, represent phase and fluorescence views of SB-1 cells grown in IB5 medium for 20 h without peptides. Mitochondrial labeling was observed at the cellular periphery, in cytoplasmic strands, and in the cytoplasm surrounding the nucleus (Fig. 1 B). Addition of up to 1 mg/ml GRGESP to growth medium followed by incubation for 20 h did not result in detectable changes in cell morphology or in the mitochondrial pattern of staining (Fig. 1, C and D). Addition of the peptide SPGDRG (0.5 mg/ml) to the growth media also failed to produce any alterations in cell morphology (data not shown). In contrast, incubation of cells with 0.25 mg/ml GRGDS for 20 h resulted in major morphological changes observed in >90% of the cells and represented in Fig. 1, E-H. A population of SB-1 cells demonstrated
Figure 1. Microscopy of soybean (SB-1 cell line) cells cultured for 20 h in IB5 medium in the absence and presence of GRGDSP or GRGESP synthetic peptides (peptides were custom synthesized by Dr. M. Pierschbacher, La Jolla Cancer Research Foundation, La Jolla, CA). Samples stained with rhodamine 123 (10 µg/ml media) for 1 min were viewed using phase-contrast optics and epifluorescent illumination. Magnification is 1200 for each figure. Phase and fluorescent views of SB-1 cells are shown in the absence of peptides (A and B); in the presence of 0.25 mg/ml GRGESP (C and D); and in the presence of 0.25 mg/ml GRGDSP (E-H). Arrows indicate plasma membrane/wall contact areas (A, B, E, and F) or plasma membrane dissociated from wall (G and H). Bar, 5 µm.
Figure 2. Microscopy of SB-1 cells cultured for 20 h under plasmolyzing conditions in modified Gamborg buffer (0.4 M sorbitol, 0.17 M glucose) in the absence and presence of GRGDSP or GRGESP synthetic peptides and then transferred back to 1B5 medium. Samples were stained with rhodamine 123 (10 μg/ml media) for 1 min and viewed using phase-contrast optics and epifluorescent illumination. Phase and fluorescent views of plasmolyzed SB-1 cells are shown in the absence of peptides (A and B); in the presence of 0.5 mg/ml GRGESP (C and D); and in the presence of 0.25 mg/ml GRGDSP (E–H). Arrows indicate plasma membrane/wall contact areas (A–D) and retracted plasma membrane (E–H). Bar, 5 μm.
aberrant cell wall organization as indicated in Fig. 1, E and F by random and incomplete cell wall formation in a giant cell. The cytoplasm was disorganized with extensive vacuolization. Another major structural abnormality observed in many cells was a detachment of the plasma membrane from the cell wall, resulting in unattached cytoplasmic masses within rigid cell walls (Fig. 1, G and H). Some of these structural rearrangements in cells grown in the presence of GRGDSP could first be observed at a concentration of 1 μg/ml (data not shown), approximately in the concentration range at which this peptide was shown to inhibit normal rat kidney cell attachment to fibronectin (38).

To more clearly examine effects on plasma membrane-wall attachment, cells were grown under mild conditions of plasmolysis for 20 h in modified Gamborg buffer containing sorbitol (0.4 M) and glucose (0.17 M). Fig. 2 (A and B) shows phase and fluorescent views of control cells labeled with rhodamine 123, while Fig. 2 (C and D) provides corresponding views of cells containing 0.5 mg/ml GRGESP. The conditions of plasmolysis enhance the visualization of cytoplasmic strands; the cytoplasm surrounding the nucleus and at the cellular periphery is heavily stained with rhodamine 123 (suggesting mitochondrial localization). In contrast, cells grown under plasmolyzing conditions in the presence of GRGDSP (0.25 mg/ml) show collapsed cytoplasmic structures within a rigid cell wall in greater than 80% of the walled cell structures viewed (Fig. 2 E), indicating a loss of plasma membrane attachment to the cell wall as observed in Fig. 1 G under nonplasmolyzing conditions. Although staining with rhodamine 123 was apparent, neither labeling of peripheral cytoplasm nor the presence of cytoplasmic strands could be clearly delineated (Fig. 2 F). Many cells grown under plasmolyzing conditions in the presence of GRGDSP (0.25 mg/ml) demonstrated multiple spherical bundles of protoplast-like structures of different sizes within a rigid cell wall (Fig. 2 G). These multiple membrane-bound structures were labeled with rhodamine 123 (Fig. 2 H) and could be observed as early as 4 h after growth initiation.

### Proliferation of Cells in RGD Peptide–Containing Media

To examine whether the RGD-containing peptide affected cell proliferation or viability, a growth curve was established for cells grown in the absence or presence of GRGDSP (0.5 mg/ml) (Fig. 3). Growth was also monitored for cells grown in the presence of the synthetic peptide SPGDRG or GRGESP (0.5 mg/ml). The growth curves in Fig. 3 monitored by wet weight indicate that cells grown either in the absence or presence of SPGDRG or GRGESP had normal doubling times of ~24–26 h. In marked contrast, these same cells cultured in the presence of GRGDSP had grown to 3–4 times their starting weight during this 24–26-h period of analysis. Supplementary analysis of DNA content for select points, and RNA content (data not shown) demonstrated a direct correspondence between the observed increase in wet weight and the increase in nucleic acids, suggesting wet weight measurements were an accurate reflection of enhanced mass rather than cell enlargement. In examining this transient proliferative burst, it is important to point out that the RGD-induced protoplast-like structures observed in Figs. 1 and 2 are more appropriately termed pseudoprotoplasts. Although not associated with a rigid wall, they do have a cellulosic component at their surface that stains with Calcofluor (Fig. 4 D). Similar pseudoprotoplast structures have previously been demonstrated to divide (14, 30–32, 45). Such divisions appear to more resemble a budding process, as suggested by Meyer and Abel (31), rather than turgor-mediated expansion. These results also imply that the impairment of normal plasma membrane/cell wall organization, observed in Figs. 1 and 2, for SB-1 cells cultured with GRGDSP did not slow down or halt cell proliferation; in fact, an opposite correlation is observed.

### Growth of SB-1 Soybean Cells in Medium Containing RGD Peptides Affects Cell Wall Organization

Considering the enhanced proliferation observed when soybean cells were grown in GRGDSP-containing medium (Fig. 3) and the apparently aberrant rigid cell wall organization noted in Figs. 1 and 2, a direct examination of the state of cell wall was initiated using the fluorescent β(1-4)glucan-specific stain Calcofluor (27, 35) (Fig. 4). Mildly plasmolyzed soybean cells (18) were stained with Calcofluor and visualized using phase and fluorescence microscopy. As observed under phase microscopy, the cells have a normal appearance (Fig. 4 A) with uniform Calcofluor staining (Fig. 4 B) throughout the cell. More intense staining was observed at the division sites between cells (Fig. 4 B). In contrast, cells grown in the presence of GRGDSP again demonstrated apparent plasma membrane separation from any contact with cell wall (Fig. 4 C) and, of particular interest, little wall localized Calcofluor staining (Fig. 4 D). Instead, staining appears punctate on the plasmolyzed protoplast surface (Fig. 4 D). It would appear that the organization and synthesis of
Figure 4. Microscopy of SB-I cells after 20 h of growth in the absence and presence of GRGDSP and resuspension in modified Gamborg buffer (0.4 M sorbitol, 0.17 M glucose). Samples were stained with 0.1% Calcofluor in 0.4 M sorbitol for 1 min. Phase and fluorescent views of plasmolyzed SB-I cells are shown that were grown in the absence of peptide (A and B) and in the presence of 0.25 mg/ml GRGDSP (C and D). Arrows indicate plasmolyzed cells (A and C) and Calcofluor cell wall staining (B and D). Bar, 5 μm.

Figure 5. Immunological analysis of anti-rat liver connexin antibody binding to samples resolved by SDS-PAGE (acylamide composition 12.5%) and transferred to nitrocellulose (400 mA for 90 min). Samples were diluted 1:1 (vol/vol) with β-mercaptoethanol sample buffer and were allowed to stand 30 min at room temperature. Primary antibody was employed at a 1:100 dilution. Antibody binding was detected here and elsewhere using alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO) according to the manufacturer's instructions. The lanes contained conditioned media grown in: (lane A) the absence of synthetic peptides; (lane B) the presence of 0.25 mg/ml GRGESP; (lane C) the presence of 0.25 mg/ml GRGDSP; (lane D) the presence of 0.25 mg/ml GRGDSPK. Lanes E and F contained soybean and rat liver connexin fraction, respectively.

The cellulose component of cell wall is considerably affected by GRGDSP. Growth of cells in GRGESP or SPGDRG peptides demonstrated Calcofluor staining indistinguishable from control cells (data not shown). The morphological and growth data taken together provide evidence that the plant cell membrane/cell wall organization and development are severely disrupted when plant cells are grown in the presence of RGD-containing peptides. A concomitant enhancement of nuclear division and proliferation is found to occur.

**RGD-Mediated Release of Soybean Connexin from SB-I Cells**

Previous reports from our laboratory provided evidence for the existence of a rat liver connexin homologous polypeptide located at the plasma membrane/cell wall interface (28). This polypeptide (soybean connexin) was suggested to be an element of a putative soybean connexon that can form the trans-wall structure termed the plasmodesma, the putative intercellular communication channel (4). In view of their suggested localization at the membrane/wall interface and possible sensitivity to perturbations of normal cell wall organization, we pursued an examination of the effects of cell growth in RGD peptides on soybean connexin integration. Fig. 5 shows an immunoblot probed with anti-rat liver con-
Figure 6. A 70–72-kD protein from SB-1 cells binds an immobilized Arg-Gly-Asp-containing peptide. A detergent extract from SB-1 protoplasts was allowed to bind an affinity column consisting of Gly-Arg-Gly-Asp-Ser-Pro-Lys-Sepharose as described in the Materials and Methods section. The column was extensively washed and individual fractions (1 ml) were collected and concentrated 10-fold before (lane 2) and after (lane 3) elution in the presence of soluble Gly-Arg-Gly-Asp-Ser-Pro. Samples (20 μl) were analyzed by SDS-PAGE under nonreducing conditions on 7.5% polyacrylamide gels. Molecular weight was determined based upon the migration of the following standards (lane 1): myosin, 200 kD; β-galactosidase, 116 kD; phosphorylase b, 97 kD; BSA, 67 kD; and ovalbumin, 45 kD.

Soybean Cells Contain an RGD Binding Protein

The morphological and proliferative modifications observed for soybean cells grown in the presence of synthetic peptides suggested that an RGD recognition system based on an RGD binding protein may be involved in plant growth and membrane/wall organization. To examine such a possibility, detergent extracts (β-octylglucoside) of SB-1 cells were allowed to bind to a column of Gly-Arg-Gly-Asp-Ser-Pro-Lys-Sepharose that was then washed and eluted with soluble GRGDSP. This procedure was previously used to isolate the hVNR, an RGD-dependent receptor from human endothelial and melanoma cells (9, 10) and placenta (41). As observed in Fig. 6 (lane 3), a 70–72-kD polypeptide is eluted from the column in the presence of the soluble GRGDSP peptide (0.1 mg/ml). Before peptide addition, no 70–72-kD polypeptide release could be detected (lane 2). In a control experiment, an aliquot of the same lysate was allowed to incubate on a column containing Sepharose alone. In this case, the 70–72-kD protein was not retained (data not shown). It should, however, be pointed out that, unlike in vivo measurements demonstrating specific RGD-dependent morphological and growth modifications, other peptides of identical composition but different sequence are also capable of eluting the 70–72-kD protein from the affinity matrix. This suggests that the interaction between the detergent-isolated 70–72-kD protein and the RGD-containing matrix is of low affinity. This may not be surprising since the conditions used in this experiment were optimized for the isolation of an RGD-containing receptor from human, not plant, cells (41) that may, to some extent, adversely affect the plant protein affinity.

Immunological Recognition of the SB-1 70–72-kD Polypeptide by an Antibody Directed to the β Subunit of a Member of the Integrin Family of Cell Adhesion Receptors

To determine whether SB-1 cells contain a molecule immunologically related to a member of the integrin family of cell adhesion receptors that are expressed by a wide variety of animal cells, immunoblotting experiments were performed. Both total SB-1 cell lysates and the RGD affinity-purified protein were analyzed on nonreduced SDS-PAGE gels that were transferred to nitrocellulose and probed with polyclonal antisera directed to the human fibronectin or vitronectin receptors. As shown in Fig. 7, the antiserum directed to the VNR recognized a 70–72-kD band in the total cell lysate (lane 1) and in the material eluted from the RGD affinity column (lane 2). This antiserum primarily recognizes the β subunit of the vitronectin receptor isolated from human placenta (lane 3). The purified placental vitronectin receptor, shown in lane 4, is visualized by Coomassie blue stain and demonstrates the α and β chain. These immunological results were mimicked by monospecific antibody to the β subunit of hVNR (Meiners, S., unpublished results, data not shown). Monospecific antibody was prepared as described (47). In contrast, polyclonal antisera directed to the fibronectin receptor failed to recognize the SB-1 proteins (lanes 5 and 6) or the purified vitronectin receptor (lane 7), although specifically reacting with the fibronectin receptor from a number of cultured human cell lines (data not shown).

Immunolocalization of 70–72-kD Polypeptide in Soybean Cells

To examine the cellular localization of the putative plant RGD binding protein (70–72-kD polypeptide), anti-hVNR antiserum, in conjunction with rhodamine-labeled secondary antibody, were utilized. Whole cells were labeled as described in Methods and viewed using phase and epifluorescent optics. Figs. 8, A and B, are phase and fluorescent views of whole cells, respectively, labeled with anti-hVNR and rhodamine-labeled secondary antibody. Little fluorescence is observed with the intact cell wall (Fig. 8 B). This is comparable to the control with preimmune serum (Fig. 8 D). Fig. 8 C is the phase view of the preimmune control. In stark con-
Figure 7. Immunoblots of B3-related protein in SB-1 soybean cells. Extracts of SB-1 cells (lanes 1 and 5, ~75 μg/lane), GRGDSP eluted material (lanes 2 and 6, ~1 μg/lane), and intact placental vitronectin receptor (VNR) (lanes 3, 4, and 7, ~2 μg/lane) were separated by SDS-PAGE under nonreducing conditions, transferred to Immobilon (Millipore Continental Water Systems, Bedford, MA) and probed with anti-VNR antiserum (lanes 1-3) or anti-fibronectin receptor (FNR) antiserum (lanes 5-7). The total VNR (lane 4) was visualized by Coomassie blue staining of the Immobilon membrane. Arrows marked the relative molecular masses based on the migration of standards as shown in Fig. 5.

Contrast, when cells are mildly treated with pectinase to enhance antibody accessibility across the cell wall (3), punctate patches of strong fluorescence are observed at the cellular periphery (Fig. 8 F), particularly at the cell wall/membrane interface. The phase view of these cells demonstrates that the pectinase treatment does not alter overall cellular morphology (Fig. 8 E). Fig. 8, G and H, represent the preimmune control of pectinase-treated cells. A background level of labeling of cells is observed (Fig. 8 G). To further pursue localization, cells were plasmolyzed and then treated with pectinase (Fig. 9 A). Strong plasma membrane fluorescence was observed following labeling with anti-hVNR and fluorescent secondary antibody, providing good evidence that the 70-72-kD polypeptide is predominantly located in the plasma membrane (Fig. 9 B). Plasmolyzed and pectinase-treated cells (Fig. 9, C and D) labeled with preimmune serum demonstrated no fluorescence (Fig. 9 D). It was also determined by immunoblot analysis that no cross-reactivity exists between pectinase and anti-hVNR (data not shown). It is interesting to note that a membrane localized randomization of the 70-72-kD antigen may occur after plasmolysis and cell membrane/wall detachment (compare Fig. 8 F and 9 B).

Discussion

RGD-dependent cell adhesion constitutes a versatile recognition system providing cells with anchorage, traction for migration, signals for polarity, position, differentiation, and possibly growth (44). Except for the process of cellular locomotion, this description may equally well define the role of

Figure 8. Fluorescence immunolocalization of 70-72-kD polypeptide in soybean cells. Cells were treated with rabbit anti-hVNR antiserum (A, B, E, and F) or preimmune serum (C, D, G, and H) followed by rhodamine-conjugated goat anti-rabbit immunoglobulin (Boehringer Mannheim Biochemicals, Indianapolis, IN). A-D represent phase and fluorescent views of SB-1 cells, while E-H represent phase and fluorescent views of SB-1 cells after cell wall permeabilization by 0.1 mg/ml pectinase (4). Bar, 5 μm.
Figure 9. Fluorescence immunolocalization of 70-72-kD polypeptide in plasmolyzed soybean cells. Cells were treated with rabbit anti-hVNR antiserum (A and B) or preimmune serum (C and D) followed by rhodamine conjugated goat anti-rabbit immunoglobulin. Cell walls were permeabilized by pectinase and plasmolyzed in modified Gamborg buffer (0.4 M sorbitol, 0.17 M glucose). Bar, 5 µm.

The plasma membrane-ECM interactions vital for anchorage-dependent growth in animal cells may be representative of mechanistic and structural antecedents in plant cells. Structures facilitating interactions between plant membrane and cell wall for the integration and coordination of cytoplasmic and nuclear division with cell wall growth may have evolved to control similar types of cooperative activity between animal cell membrane and ECM. Anchorage-dependent growth in this manner could be considered a common requirement for animals and plants. Morphological evidence presented in this study finds a direct correlation between the requirement for the RGD sequence in synthetic peptides and their disruptive influence on control and coordination of normal cell wall/plasma membrane organization and development during growth. This is demonstrated by RGD peptide specific: (a) plasma membrane detachment from cell wall; (b) gross morphological disorganization of cell wall; (c) radically altered distribution of cellulose as observed by Calcofluor staining; (d) release of soybean connexin during growth; and (e) significantly enhanced proliferation.

Evidence that these RGD-related phenomena are a result of the existence and activity of a plant RGD binding protein may be suggested for the following reasons: (a) by analogy with animal cells, RGD peptides may interfere with the physical coupling of plasma membranes to adhesive glycoproteins containing the RGD sequence; no other RGD-related intracellular mechanisms have been reported; (b) immunological cross-reactivity has been observed between polyclonal anti-hVNR (recognizing the B3 subunit in VNR and platelet adhesion receptor GPIIb/IIIa; [40]) and a 70-72-kD polypeptide extracted from soybean plasma membrane; (c) a 70-72-kD polypeptide cross-reactive with anti-hVNR antibody is retained on a GRGDSPK-Sepharose column; (d) immunolocalization demonstrates that the 70-72-kD polypeptide is found in patches at the cell wall/membrane interface and is predominantly a component of the plasma membrane; and (e) recent work demonstrates the presence in soybean seeds of low molecular weight polypeptides showing a fibronectin-related stretch of sequence containing RGD (36) that could serve as ligands in the cell wall for plasma membrane-localized plant RGD binding protein(s).

At present, it is not clear whether the SB-1 cell β-like subunit is part of a heterodimeric complex, as observed for members of the integrin family of adhesion receptors in animal cells. Although our RGD affinity experiments failed to demonstrate the presence of any other polypeptide, it is possible, once solubilized, they easily dissociate. It is conceivable that the 70-72-kD plant protein is similar to a 68-kD...
RGD binding protein from the slime mold Dictostelium, which also undergoes RGD-dependent adhesive interactions (13). However, our anti-VNR antibody did not react with the Dictostelium protein (Springer, W., personal communication).

**Attachment Mediated Control of Growth: An Evolutionary Argument**

Although plasma membrane/wall associations in plant cells are clearly observed after plasmolysis, no literature appears to exist examining the mechanism or role of such attachments. Such membrane/wall associations have, however, been extensively explored in bacteria. In fact, an essential element in the theory for bacterial growth as initially presented by Jacob (23) is the integration of the processes of DNA replication, cell wall biosynthesis, and cell division through a physical linkage of DNA to the cell wall, presumably through the intervention of the cytoplasmic membrane. Subsequent experiments have demonstrated the controlling influence of cell wall biosynthesis on the organization of bacterial cell shape and growth (33, 49), and the assembly and maintenance of polar surface structures. This latter aspect is most dramatically represented by flagellum and phage receptor site polar localization (46, 48). The molecular mechanism of coordination between wall and plasma membrane has remained elusive. Recent reports have demonstrated a fibronectin binding molecule in S. aureus (12), while we observed that immunobLOTS of detergent extracts from Escherichia coli (K-12) and Saccharomyces cerevisiae (baker's yeast), probed with anti-VNR antibody, demonstrated single bands in the 70–80-kD region (Meiners, S., unpublished results). Such immunologically related integrin type homologues may be elements of membrane wall attachment mechanisms in bacteria and yeast serving as elements of the observed zones of adhesion between plasma membrane and wall (5, 6). It may be suggested that the phenomena of cytoplasmic membrane–wall associations in bacteria, cell wall–plasma membrane interactions in plants, and integrin–ECM attachment in animal cells are all mechanistically linked through a common requirement for membrane attachment to an organized complex polysaccharide proteoglycan polymer that serves to promote coordination of unified cellular compartmental growth. This attachment may also function to project such intracellular organization and communication to the more complex cell–cell level.

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**References**

1. Albersheim, P. 1976. The primary cell wall. In *Plant Biochemistry*. J. Bonner and J. E. Varner, editors. Academic Press Inc., New York. 225–273.
2. Arrunt, D. R., H. A. Kaplan, H. Moyer, and W. J. Leenstar. 1986. The effects of hexapeptides on attachment and outgrowth of mouse blastocysts cultured in vitro: evidence for the involvement of the cell recognition tripeptide Arg-Gly-Asp. *Proc. Natl. Acad. Sci. USA.* 83:6751–6755.
3. Baron-Epel, O., D. Hernandez, L.-W. Jiang, S. Meiners, and M. Schindler. 1988. Dynamic continuity of cytoplasmic and membrane compartments between plant cells. *J. Cell Biol.* 106:715–721.
4. Baron-Epel, O., P. K. Gharyal, and M. Schindler. 1988. Pectins as mediators of wall porosity in soybean cells. *Planta (Berl.)* 175:389–395.
5. Bayer, M. E. 1968. Arrangement of wall and membrane of Escherichia coli. *J. Gen. Microbiol.* 53:395–404.
6. Bayer, M. E. 1979. The fusion sites between outer membrane and cytoplasmic membrane of bacteria: their role in membrane assembly and virus infection. In *Bacterial Outer Membranes: Biogenesis and Functions*. M. Inouye, editor. John Wiley & Sons Inc., New York. 167–202.
7. Boucard, J., T. Darrabere, T. J. Poole, H. Aoyama, K. Yamada, and J. P. Thiery. 1984. Biologically active synthetic peptides as probes of embryonic development: a competitive peptide inhibitor of fibronectin function inhibits gastrulation in amphibian embryos and neural crest migration in avian embryos. *J. Cell Biol.* 99:1822–1831.
8. Burton, K. 1966. Determination of DNA concentration with diphenylamine. *Methods Enzymology.* 12(B):163–166.
9. Cheresh, D. A. 1987. Human endothelial cells synthesize and express an Arg-Gly-Asp-directed adhesion receptor involved in attachment to fibronectin and von Willebrand factor. *Proc. Natl. Acad. Sci. USA.* 84:6471–6475.
10. Cheresh, D. A., and J. R. Harper. 1987. Arg-Gly-Asp recognition by a cell adhesion receptor requires its 130 kDa subunit. *J. Biol. Chem.* 262:1434–1437.
11. Dethard, S. E. Ruoosihi, and M. D. Pierschbacher. 1987. A cell surface receptor complex for collagen Type I recognizes the Arg-Gly-Asp sequence. *J. Cell Biol.* 104:585–593.
12. Flock, J.-l., G. FrSman, K. J6nsson, B. Guss, C. Signis, B. Nilsson, G. Raucci, M. H6l6k, T. Wadstr6m, and M. Lindberg. 1987. Cloning and expression of the gene for a fibronectin-binding protein from *Staphylococcus aureus*. EMBO (Eur. Mol. Biol. Organ.) J. 6:2351–2357.
13. Gabius, H.-J., W. R. Springer, and S. H. Barondes. 1985. Receptor for the cell binding site of Discoidin I. *Cell.* 42:469–480.
14. Hahn, G., and Hoffman, F. 1984. Dimethyl sulfoxide can initiate cell divisions of arrested callus protoplasts by promoting cortical microtubule assembly. *Proc. Natl. Acad. Sci. USA.* 81:5449–5453.
15. Hynes, R. O. 1987. Integrins: a family of cell surface receptors. *Nature* 324:565–570.
16. Hay, E. D. 1981. Extracellular matrix. *J. Cell Biol.* 91:205–223a.
17. Hayman, E. G., M. D. Pierschbacher, Y. Ohgren, and E. Ruoosihi. 1983. Serum spreading factor (vitronectin) is present at the cell surface and in tissues. *Proc. Natl. Acad. Sci. USA.* 80:4003–4007.
18. Hayman, E. G., S. C. Malek-Hedayat, J. L. Wang, and M. Schindler. 1986. Endogenous lectins from cultured soybean cells: isolation of a protein immunologically cross-reactive with seed soybean agglutinin and analysis of its role in binding of *Rhizobium japonicum*. *J. Cell Biol.* 103:1043–1054.
19. Hughes, M. J., K. Olden, and K. M. Yamada. 1986. A synthetic peptide from fibronectin inhibits experimental metastasis of murine melanoma cells. *Science (Wash. DC)* 233:467–470.
20. Hynes, R. O. 1987. Integrins: a family of cell surface receptors. *Cell.* 48:549–554.
21. Hynes, R. O., and K. M. Yamada. 1982. Fibronectins: multifunctional modular glycoproteins. *J. Cell Biol.* 95:369–377.
22. Ignoffo, R. A., and J. Massagué. 1987. Cell adhesion protein receptors as growth factors for transformed cells. *Science.* 235:189–197.
23. Jacob, F., S. Brenner, and F. Cuzin. 1963. On the regulation of DNA replication in bacteria. *Blood.* 66:946–956.
24. Johnson, L. V., M. L. Walsh, and L. B. Chen. 1980. Localization of mitochondria in living cells with rhodamine 123. *Proc. Natl. Acad. Sci. USA.* 77:990–994.
25. Karr, M. E., and G. N. Wadstr6m. 1987. Cloning and expression of the cell binding site of Discoidin I. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:2351–2357.
26. Leptin, M., R. Aebersold, and M. Wilcox. 1987. Drosophila position-specific antigens resemble the vertebrate fibronectin-receptor family. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:1037–1043.
27. Liotta, L. A. 1986. Tumor invasion and metastasis: role of the extracellular matrix. *Cancer Res.* 46:1–7.
28. Maeda, H., and N. Ishida. 1967. Specificity of binding of hexopyranosyl polysaccharides with fluorescent brightener. *J. Biochem. (Tokyo).* 62:276–278.
29. Meiners, S., and M. Schindler. 1987. Immunological evidence for gap junction polypeptide in plant cells. *J. Biol. Chem.* 262:951–953.
30. Miettinen, T., N. Illin, J. L. Wang, K. R. Schobert, and M. Schindler. 1983. Lecin receptors on the plasma membrane of soybean cells. Binding and lateral diffusion of lectins. *Biochemistry.* 22:396–3975.
31. Meyer, Y., and W. D. Abel. 1975. Importance of the wall for cell division and in the activity of the cytoplasm in cultured tobacco protoplasts. *Planta (Berl.)* 123:33–40.
32. Meyer, Y., and W. O. Abel. 1975. Budding and cleavage division of tobacco mesophyll protoplasts in relation to pseudo-wall and wall formation. *Planta (Berl.)* 125:11–15.
33. Meyer, Y., and W. Herbst. 1978. Chemical inhibition of cell wall formation and cytokinesis, but not of nuclear division in protoplasts of *Nicotiana tabacum* L. cultured in vitro. *Planta (Berl.)* 142:253–262.
34. Mirelman, D. 1979. Biosynthesis and assembly of cell wall peptido-glycan. In *Bacterial Outer Membranes: Biogenesis and Functions*. M. Inouye, editor. John Wiley & Sons Inc., New York. 115–116.

The Journal of Cell Biology, Volume 108, 1989
34. Munro, H. N., and A. Fleck. 1965. The determination of nucleic acids. Methods Biochem. Anal. 14:113–176.
35. Nagata, T., and I. Takebe. 1970. Cell wall regeneration and cell division in isolated tobacco mesophyll protoplasts. Planta (Berl.). 92:301–308.
36. Odani, S., T. Koide, and T. Ono. 1987. Amino acid sequence of a soybean (Glycine max) seed polypeptide having a poly-(L-aspartic acid) structure. J. Biol. Chem. 262:10502–10505.
37. Pierschbacher, M. D., and E. Ruoslahti. 1984. Cell attachment activity of fibronectin can be duplicated by small synthetic fragment of the molecule. J. Biol. Chem. 262:10502–10505.
38. Pierschbacher, M. D., E. G. Hayman, and E. Ruoslahti. 1983. Synthetic peptides with cell attachment activity of fibronectin. Proc. Natl. Acad. Sci. USA. 80:1224–1227.
39. Pierschbacher, M. D., and E. Ruoslahti. 1984. Variants of the cell recognition site of fibronectin that retain attachment-promoting activity. Proc. Natl. Acad. Sci. USA. 81:5985–5988.
40. Plow, E. F., M. D. Pierschbacher, E. Ruoslahti, G. A. Marguerie, and M. H. Ginsberg. 1985. The effect of Arg-Gly-Asp-containing peptides on fibronectin and von Willebrand factor binding to platelets. Proc. Natl. Acad. Sci. USA. 82:8057–8061.
41. Pytela, R., M. D. Pierschbacher, and E. Ruoslahti. 1985. A 125/115 kD cell surface receptor specific for vitronectin interacts with the Arginine-Glycine-Aspartic acid adhesion sequence derived from fibronectin. Proc. Natl. Acad. Sci. USA. 82:5766–5770.
42. Schindler, L. 1977. Role of the cell wall in the ability of tobacco protoplasts to form callus. Planta (Berl.). 135:177–181.
43. Shapiro, L. 1985. Generation of polarity during Caulobacter cell differentiation. Annu. Rev. Cell Biol. 1:173–207.
44. Smith, D. E., and P. A. Fisher. 1984. Identification, developmental regulation, response to heat shock of two antigenically related forms of a major nuclear envelope protein in Drosophila embryos: application of an improved method for affinity purification of antibodies using polypeptide immobilized on nitrocellulose blots. J. Cell Biol. 99:20–28.
45. Sommer, J. M., and A. Newton. 1988. Sequential regulation of developmental events during polar morphogenesis in Caulobacter crescentus: assembly of pili and swarmer cells requires cell separation. J. Bacteriol. 170:409–415.
46. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA. 76:4350–4354.
47. Vannini, G. L., S. Pancaldi, F. Poli, and M. P. Fasulo. 1988. Rhodamine 123 as a vital stain for mitochondria of plant cells. Plant Cell Environ. 11:123–127.