Integrin-Like Proteins in the Pollen Tube: Detection, Localization and Function

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The distribution of integrin-like proteins in the pollen tube was examined by immunofluorescence labeling and western blotting techniques using antibodies against human placenta integrin vitronectin receptor (VnR), and α, β, and β integrin subunits. Pseudocolor-coded confocal images showed intense immunostaining within 10 and 5 μm of the tip of the pollen tube in Lilium davidii and Nicotiana tabacum respectively. In both segments the site near the plasma membrane was labeled. Western blotting analyses revealed cross-reaction of anti-β, anti-α, and anti-VnR with the proteins in the plasma membrane preparation of L. davidii and Hemerocallis citrina pollen tube. These studies provide evidence for the first time that the integrin-like protein is present in pollen tubes, and it may be mainly composed of α and β subunits in lily pollen tubes. In a functional assay, neither anti-VnR antibody nor the Arg-Gly-Asp-Ser tetrapeptide inhibited pollen tube growth of N. tabacum in vitro, but both of them depressed tube growth on the stigma and in style under quasi in vivo culture conditions. The integrin-like proteins localized in the tip and periphery of the pollen tube appeared to play roles in growth of the pollen tube tip and interaction with the extracellular matrix of the style.

Key words: Immunochemical detection/localization --- Integrin-like protein (subunits) --- Pollen tube --- Tip growth.

Abbreviations: BK medium, Brewback and Kwack medium; ECM, extracellular matrix; FITC, fluorescein isothiocyanate; PM, plasma membrane; RGDS, Arg-Gly-Asp-Ser; RGES, Arg-Gly-Glu-Ser; Vn, vitronectin; VnR, vitronectin receptor.

Introduction

Integrins, a large family of transmembrane proteins in animal cells, exist as heterodimers of α and β subunits. Serving as receptors for extracellular matrix (ECM) proteins including fibronectin (Fn), vitronectin (Vn), laminin (Ln) and collagen (Hynes 1987), they link the ECM to the cytoskeleton through their extracellular N-terminal domains and their cytoplasmic C-terminal domains. Integrins mediate both outside-in and inside-out signaling, and are involved in numerous processes such as motion, differentiation, and growth of cells (Clark and Brugge 1995) in the animal system.

Schindler et al. (1989) showed that integrin-like proteins are present in plant material—suspension cultured soybean root cells. Some recent studies revealed the existence of integrin-like proteins in several other species of plant cells, e.g., onion epidermal cells (Gens et al. 1996), Arabidopsis cells (Katemb et al. 1997, Faik et al. 1998), epicotyl cells of Pisum sativum (Kiba et al. 1998), and maize calluses (Labouré et al. 1999). However, it has not yet been identified in pollen or pollen tubes (Li et al. 1997). Pollen germination and tube growth are important events during sexual reproduction in higher plants. Some stylar components can attract the pollen tube and accelerate its growth (Cheung et al. 1995). Using polyclonal antibodies in animals against vertebrate vitronectin, Sanders et al. (1991) immunolocalized Vn-like protein, which is a matrix protein and usually binds to Vn receptor (VnR), on the surface of stylar transmitting tissue cells. The ‘actin filament-organizing sites’ were also detected in the pollen tube in vivo (Li et al. 1997). These findings imply that there may be a linker on the plasma membrane of cells in the pollen tube responsible for the interaction between stylar ECM and cytoskeleton of the pollen tube.

In the present study, we detected and immunolocalized integrin-like proteins in the pollen tube, and determined the composition of integrin subunits by immunofluorescence labeling and western blotting techniques. We also studied the possible role of integrin-like proteins during pollen tube growth using antibody against VnR and the Arg-Gly-Asp-Ser tetrapeptide (RGDS) in both in vitro and quasi in vivo conditions.

Materials and Methods

Plant materials

Pollen of Lilium davidii Duch and Hemerocallis citrina Baroni were collected and stored at –70°C until use. After thawing and rehydration for 10 h at 4°C and 2 h at room temperature in a moist chamber, the pollen were cultured in Brevbaker and Kwack (1963) medium (BK medium) containing 15% (w/v) sucrose. Fresh pollen of Nicotiana tabacum was directly cultured in BK medium containing 10% (w/v) sucrose at 25°C for 3–4 h; then pollen tubes were collected.

Antibodies and agents

Polyclonal antibodies raised in rabbits against human placenta integrin VnR and against the cytoplasmic domain of β, β, α integrin subunits were a kind gift from Dr. Z.H. Zhang (La Jolla Cancer Research foundation, La Jolla, CA, U.S.A.). Tetrapeptides RGDS (#A-
Isolation of pollen tube plasma membrane

The plasma membrane (PM) of the pollen tube was prepared as described by Noguchi and Morre (1991) with some modifications. Pollen tubes were collected, ground and sonicated (10 × 20 s) in 25 mM Tris-MES buffer (pH 7.6), containing 0.25 M sucrose, 2 mM EGTA, 2 mM MgCl₂, 0.2% bovine serum albumin (BSA), 0.2% casein, 10 mM β-glycerolphosphate disodium, 10% (v/v) glycerol, 2 mM ATP, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (Chen and Wang 1994) at 4°C with a Sonifier Cell Disruptor until pollen tubes were totally disrupted. The homogenates were centrifuged for 30 min at 9,000 × g to remove cell debris, nuclei, and part of mitochondria. The resultant supernatant was then centrifuged at 100,000 × g for 1 h to yield microsomal pellets. PM was isolated from the microsomal pellets by two-phase partitioning. The final concentrations of components of the two-phase system were 6.4% (w/w) Dextran T-500 (Pharmacia), 6.4% (w/w) polyethylene glycol 3350 (Sigma). The upper phase, enriched in PM, was collected and diluted (1 : 5) with 5 mM potassium phosphate buffer (pH 7.8) containing 0.25 M sucrose, and then centrifuged at 100,000 × g for 1 h at 0°C. Final pellets were collected and used for western blotting analysis. The purity of PM was examined as described by Larsson et al. (1987) and by staining with low-pH phosphotungstic acid (Roland et al. 1972).

Electrophoresis and western blotting analysis

PM was suspended and boiled in sample buffer, then subjected to SDS-PAGE with a 4–10% gradient gel according to the method of Laemmli (1970). About 40 µg protein were loaded onto each lane. The proteins were transferred to nitrocellulose membrane according to the method of Towbin et al. (1979) for western blotting analysis. Then, nitrocellulose membrane was first blocked with 1% (w/v) BSA in TBST (Tris-Cl buffer, pH 7.4, containing 150 mM NaCl, 0.05% Tween-20), then incubated with primary integrin antibodies (dilution 1 : 100) at 4°C overnight, and secondary goat anti-rabbit IgG antibody conjugated to alkaline phosphatase (Sigma; dilution: 1 : 20,000) at room temperature for 2 h. The color was developed with 5-bromo-4-chloro-3-indolyl phosphate and p-nitroblue tetrazolium (Sigma) according to the manufacturer’s instructions. Non-immune serum and secondary antibody alone were used in control experiments.

Quasi in vivo experiment

(i) Pollen tube growing on stigma: Stigmata of N. tabacum were immersed in BK medium (pH 6.0) containing RGDS or RGES for 2.5 h before pollination with fresh pollen. Then stigmata were cultured in a moist chamber at 25 ± 1°C for 1.5 h, fixed in Carnoy’s solution, softened in 5 M NaOH, rinsed, and then stained with 1.6% Aniline Blue. The length of pollen tubes was measured under a microscope. (ii) Pollen tube growth in the style: Unpollinated pistils of N. tabacum were cut 6 mm from stigmata. Then various concentrations of anti-VnR antibody or RGDS in BK medium were microinjected into transmitting tissue of styles at the cut end using a microinjector (Nikon/Nariashige, NT88). After pollination with fresh pollen, the styles were immediately immersed into the same BK medium as used for microinjection with the cut end down. Non-immune serum and RGES were used in control experiments. We used approximately six to eight styles per group, and repeated each treatment three to four times. All styles were cultured in a moist chamber at 26°C; the time required for pollen tubes to emerge from the cut end of the style was recorded and the average growth rate of the pollen tube calculated. For those samples whose pollen tubes did not emerge from the cut end of styles, the styles were fixed in Carnoy’s solution, and softened in 5 M NaOH, then pressed with a cover slip gently to split and expose tract tissue. The pollen tubes were viewed under a microscope, the length of pollen tube was measured and growth rate calculated.

Results

Immunolocalization of integrin-like proteins in pollen tubes

To verify if there are proteins in the pollen tube that could crossreact with antibodies against human integrin, and determine their distribution, we performed indirect immunofluorescence studies. As human integrin VnR comprises α₃ and β₃ or β₇ subunits (Felding-Habermann and Cheresh 1993), we used antibodies against β₃, β₇, and α₃ integrin subunits as a primary antibodies, respectively, and FITC-conjugated secondary antibody to examine the pollen tubes of L. davidii Duch and N. tabacum by laser confocal scanning microscopy. The serial optical section images resolved by confocal microscopy revealed that integrin antibodies can recognize the antigens present in the pollen tube.

The lily pollen tube was labeled with anti-β₃ (Fig. 1) and anti-α₃ (Fig. 2) antibodies. The strongest immunofluorescence was found in the shape of a cap within 10 µm of the tip of the pollen tube. Furthermore, the fluorescence label was also found in the shank of pollen tube at a low level. The β₃ antibody produced fluorescence in the shank of the tube in a punctuate pattern distributed along the periphery of the tube (Fig. 1B), while the α₃ antibody stain, produced fluorescence in the shank of the tube in a more diffuse pattern (Fig. 2B). The immunofluorescence labeled with anti-β₃ antibody was much weaker than that labeled with α₃ and β₃ antibodies (data not shown). In the non-immune serum control, little fluorescence was observed (Fig. 2C). No autofluorescence was found in the pollen tube (data not shown), and no fluorescence after each step of treatment or staining with secondary antibody alone (data not shown). These findings indicate the specificity of the immunofluorescence.

The tobacco pollen tube was also immunostained with an antibody against the α₃ integrin subunit (Fig. 3A). The strongest fluorescence was found at the tip of the tube, in a pattern
similar to that in the lily pollen tube, but, unlike in lily, it was stained strongly with anti-β₁ antibody (Fig. 3B), but weakly with anti-β₃ antibody (data not shown). The cap-like staining was mainly located within about 5 μm of the tip of the pollen tube.

Detection of integrin-like proteins by western blotting

In order to further determine the presence of integrin-like molecules in the pollen tube and the possibility of their association with PM, we isolated PM fraction by two-phase partitioning. The purity of PM was measured by comparison with a marker enzyme, used in our laboratory (Ma et al. 1999), and by the low pH phosphotungstic acid staining under an electron microscope. The PM preparation was pure enough for our experiment (data not shown). Then, the proteins were separated by SDS-PAGE under non-reducing conditions, and analyzed by western blotting.

In the absence of reducing agent, antibodies against human β₁, αᵥ and intact VnR all recognized one band with a similar MW of ~150 kDa both in L. davidii Duch (Fig. 4, lanes 2–4) and in H. citrina Baroni (Fig. 5, lanes 3–5). We also found that the anti-β₃ antibody sometimes recognizes a minor band at 95–97 kDa in addition to the major ~150 kDa band in H. citrina Baroni under reducing conditions (Fig. 5, lane 2). No immune cross-reacting bands were observed when probed with an

Fig. 1 Pseudocolor-coded confocal images of pollen tube showing the localization of integrin-like protein by indirect immunofluorescence. L. davidii pollen tubes were labeled with anti-human β₁ integrin antibody in a cap-like pattern of fluorescence which is extremely strong in the tip region, and in a punctuate pattern of fluorescence which is relatively weak at the periphery along the length of tube. (A) Serial optical sections; (B) enlargement of No. 4 section.

Fig. 2 Pseudocolor-coded confocal images of pollen tube showing the localization of integrin-like protein by indirect immunofluorescence. L. davidii pollen tubes labeled with anti-human αᵥ integrin antibody (A, B) and non-immune serum control (C). A strong signal was also found in a cap-like pattern in the tip region and relatively weak signal was found in a more diffuse pattern in the shank of tube. Little fluorescence was observed in the control. (A) Serial optical sections; (B) enlargement of No. 2 section.

Fig. 3 Pseudocolor-coded confocal images of pollen tube showing the localization of integrin-like protein by indirect immunofluorescence. Serial optical sections of N. tabacum pollen tubes labeled with anti-αᵥ (A) and anti-β₁ (B) integrin antibodies respectively. The fluorescence patches were found at the tip of the tube.
antibody against human $\beta_1$ in either plant (data not shown). Neither with non-immune serum (Fig. 4, lane 5; Fig. 5, lane 6) nor with secondary antibody alone (data not shown) was the cross-reacting band found in control experiments.

**Effects of RGDS and anti-VnR antibody on pollen tube growth**

If integrin-like proteins are present in the pollen tube, and localized at the tube tip, or associated with plasma membrane, then what is the role they play in the pollen system?

Synthetic RGDS, an antagonist agent commonly used in detecting integrin function in animal cells, was used together with the anti-VnR antibody in both our in vitro and quasi in vivo experiments. The results showed that addition of RGDS or anti-VnR antibody directly to BK medium did not inhibit pollen tube growth in vitro (data not shown). However, the tube growth on stigmata decreased in a concentration-dependent manner by treatment of stigmata with RGDS as compared with RGES control, the maximum depression being about 36.4% (Fig. 6). The growth of the pollen tube in the style was also depressed in a concentration-dependent manner by microinjection of RGDS (Fig. 7) or anti-VnR antibody (Fig. 8) into tract tissue of styles, the maximum depression being about 37% by RGDS and 21.2% by the antibody. Since RGES and non-im-
Immune serum only slightly affected pollen tube growth, with no dose dependency (Fig. 7, 8), they did not have a specific effect. On the other hand, neither RGDS nor anti-VnR antibody totally inhibited pollen tube growth even at a concentration of 10 mg ml⁻¹ for RGDS, and 1.35 mg ml⁻¹ for antibody.

**Discussion**

The presence and distribution of integrin-like proteins in pollen tube

In this study, we found that antibodies against human integrin crossreacted with molecules present in pollen tubes of several plant species by laser confocal scanning microscopy and western blotting.

The confocal image microscopy showed that antibodies against α₉, β₁ or β₃ integrin subunits most prominently stained the tip of the pollen tube in lily (Fig. 1, 2) and tobacco (Fig. 3). This labeling pattern is in agreement with the intense staining in Arabidopsis root tips (Katembe et al. 1997) detected by immunofluorescent microscopy using an antibody against chicken β₁ integrin subunit, and a gradient was also found from the tip to the base. In our experiments, the shank of the tube was immunostained with anti-β₃ antibody along the tube periphery and in a punctuate pattern, much similar to the confocal image of Saprolegnia hyphae stained with anti-β₁ antisemur by Kaminskyj and Heath (1995). This may not be an artifact, but rather related to the pulse growth of the pollen tube (Li et al. 1992). All these similarities indicate the growth of the cells in the tip region. The present western blotting results not only confirmed the presence of integrins in the pollen tube, but also indicated that integrin-like proteins are mainly associated with the pollen tube PM.

**Subunit composition of integrin-like proteins in pollen tube**

Integrins are heterodimers of α and β subunits. To date, at least eight β subunits and 16 α subunits have been identified, comprising over 20 different classes of integrins in different types of animal cells (Clark and Brugge 1995). In the previous studies on integrin-like proteins in plants, most researchers used only one kind of integrin antibody, i.e., conservative β-integrin-subunit antibodies (Schindler et al. 1989, Gens et al. 1996, Katembe et al. 1997), which do not detect the α subunit. Recently, polyclonal antibodies against the human platelet integrin α₉β₃, and antibodies specific for either the β₁ or the α₁β₁ integrin subunit have been reported to crossreact with glycoproteins of Arabidopsis suspension cultured cells (Faik et al. 1998) and maize callus cells (Labouré et al. 1999) in western blotting, indicating that integrin-like proteins in both plants share structural similarities with the animal α₉β₃ complex.

Previously, we demonstrated by western blotting experiments that antibodies against the human VnR, and against β₃, α₉ integrin subunit cross-reacted with proteins in the PM preparation of H. citrina Baroni (Sun et al. 1998). In this study, we further demonstrated that anti-β₃, anti-α₉, and anti-VnR antibodies all recognize an ~150 kDa band both in H. citrina Baroni and in L. davisii Duch under non-reducing conditions in western blots (Fig. 4, 5). These findings suggest the presence of β₃- and α₉-related co-migrating polypeptides, and the anti-VnR antibody recognizes the epitopes present on one or two of them. Faik et al. (1998) also found co-migration of platelet β₃- and α₁β₁-related two polypeptides in Arabidopsis glycoproteins. On the other hand, anti-β₃, anti-α₉ integrin antibodies immunostained lily pollen tube (Fig. 1A, 2A). Based on all of these results, we speculate that the integrin-like protein in the lily family pollen tube may be VnR, mainly composed of α₉ and β₁ subunits.

Since the anti-β₃ antibody, but not anti-β₁, and anti-α₉ integrin antibodies immunostained the pollen tube strongly in N. tabacum, the subunit composition of integrin-like proteins in the pollen tube in tobacco may be different from that in lily.

Different types of animal cells have different integrin subunit compositions, and the subunit functions are different: the α₉ subunit mediates adhesion of the cell to ECM, while the β subunit controls the interaction between cells and extracellular proteins (Felding-Habermann and Cheresh 1993). The reason for the difference in subunit composition in different plant species is not clear. Under reducing conditions, we sometimes found the anti-β₃ antibody to crossreact with a minor band at 95–97 kDa in H. citrina Baroni in addition to a major 150 kDa band. More than one band with an antibody against chicken β₁ integrin subunit has been found in onion (Gens et al. 1996) and in Arabidopsis root (Katembe et al. 1997) under reducing conditions. Whether this phenomenon is due to the effect of reducing agent on proteins, is not clear.
Possible role of integrin-like proteins in pollen tube growth

There are few works suggesting the role of integrin-like proteins in plant cells, especially in higher plants. RGD, a tripeptide sequence in many ECM adhesion proteins of animals, is used as an antagonist to study the function of integrins. In plants, the interaction between ECM and integrin-like proteins is also thought to be RGD dependent (Wyatt and Carpita 1993). RGD-containing peptides enhance proliferation, disrupt cell-wall/plasma membrane connection in soybean cells in suspension culture (Schindler et al. 1989) and Arabidopsis cells (Faik et al. 1998), induce important morphological changes in maize callusses cell (Labouré et al. 1999), and are implicated in plant defense responses (Kiba et al. 1998). Lord and Sanders (1992) hypothesized that integrin-like proteins participate in pollen-tube extension, and proposed a model for integrin regulating the movement of pollen tube through the interaction with Vn-like proteins in the ECM of style, but did not provide experimental support.

We used RGDS and anti-VnR antibody to examine the role of integrin in a pollen system. Addition of RGDS or the anti-VnR antibody to culture medium (in vitro experiment) has no obvious inhibitory effects on pollen tube growth. This may be the result of absence of ECM secreted from the pistil in vitro, thus no interaction between the pollen tube and ECM. If this were true, then RGDS and anti-VnR antibody must affect pollen tube growth in vivo. Our quasi in vivo experiments confirmed this point. RGDS inhibited pollen tube growth (Fig. 6) on the stigma, indicating that RGDS disrupts the interaction between the pollen and stigma, whereas as usual the components secreted by the stigma can promote pollen tube growth. Secondly, microinjection of RGDS (Fig. 7) or anti-VnR antibody (Fig. 8) into the tract tissue of the style also depressed the growth of the pollen tube in a concentration-dependent manner. Thus, integrin-like proteins distributed at the tip and periphery of the pollen tube may be involved in the regulation of growth of the pollen tube tip, at the same time our results also support the model of Lord and Sanders (1992) as above mentioned.

On the other hand, neither the antibody nor RGDS totally inhibited pollen tube growth. This may be because the regulation of pollen tube growth in vivo is complicated, and the pathway mediated by integrin-like proteins might regulate pollen tube growth.

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References


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