RGD-dependent growth of maize calluses and immunodetection of an integrin-like protein

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Abstract When maize calluses are grown in the presence of the RGD peptide, important morphological changes are observed indicating the presence of a likely RGD-binding receptor. Polyclonal antibodies generated against the human β1 integrin subunit, the platelet integrin αIIbβ3 (P23) and antibodies specific for either the β3 platelet chain or the αIIb polypeptide cross-react with glycoproteins in Western blot analyses. Immunoprecipitation assays indicate that this maize integrin-like protein shares structural similarities with the animal αIIbβ3 complex. We also show that AcAt2, a polyclonal antibody raised against *Arabidopsis* proteins purified on an RGD column, interacts with a maize protein.

Key words: Plant biology; Cell wall; Plasma membrane; Receptor; Integrin; *Zea mays*

1. Introduction

In plants, much progress has been made in understanding the complexity of the cell wall chemistry [1–4] and cell wall extension [5], but there is still little information concerning its dynamic nature and the connection between it and the plasma membrane [6–8]. The plant cell wall may interact with extrinsic and intrinsic proteins of the plasma membrane, like the extracellular matrix (ECM) in animals, and these in turn may interact directly or indirectly with the cytoskeleton [9–11].

One important family of molecules involved in the ECM-plasma membrane-cytoskeleton continuum in animal cells is a family of divalent cation-dependent receptors, termed integrins [12]. Integrins are a family of heterodimeric transmembrane proteins formed by the non-covalent association of one polypeptide of 15 structurally related α subunits with one polypeptide of eight structurally related β subunits [12]. Integrins function as cell surface receptors for proteins of the extracellular matrix, or for proteins on the surface of other cells. Plasma membrane-ECM contacts are mediated by substrate adhesion molecules such as fibronectin, vitronectin, collagen, and laminin, which bind to integrins that interact in turn with the cytoskeleton. The ECM molecules often bind to integrins via the short amino acid sequence Arg-Gly-Asp (RGD). This specific recognition between cell and ECM has been implicated in cell migration [13,14], transmembrane signalling events [15,16], differentiation [17], response to hormones and growth factors [18]. In addition, integrins have been implicated in the attachment of certain bacteria and viruses to host cells [19,20].

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Evidence for the presence of integrin-like proteins in plants relies on the use of exogenous RGD-containing peptides and cross-reactivity of plant proteins with polyclonal antibodies raised against subunits of the animal receptor. Here we present data concerning the effect of RGD peptides on maize callus growth and immunological and biochemical evidence for the presence of an integrin-like protein in maize callus cells.

2. Materials and methods

2.1. Plant materials

Embryogenic maize calluses, a gift from Dr. Charles Amstrong (Monsanto Agricultural company, St. Louis, MO, USA) have been previously described [21]. Calluses were grown in type II culture medium N6 1-100-25-Ag supplemented with 2,4-D (1 mg/l). Cultures were incubated in the dark at 28°C with subculture every 3 weeks.

2.2. Antibodies and reagents

Rabbit anti-integrin β1 subunit (Chemicon, Euromedex) was raised against a synthetic peptide derived from the COOH-terminal sequence (cytoplasmic domain) of the human β1 integrin subunit [22]. P23, the antibody against the human platelet receptor αIIbβ3 used in this study, has been previously described [23]. P23 is a rabbit polyclonal antiserum raised against a purified preparation of human platelet integrin αIIbβ3 and further purified by immunoaffinity on the αIIbβ3 complex using a standard protocol [24]. Ly7 and CD61 are polyclonal rabbit antisera raised against the purified platelet β3 polypeptide and ZACA is a rabbit polyclonal antibody raised against the recombinant peptide αIIb, positions 171–464. CS3, a monoclonal antibody (mAb) purchased from Hemeris (Grenoble), is an anti-αIIbβ3 complex antibody, which does not react with the dissociated complex [25]. AcAt2, raised against *Arabidopsis* glycoproteins purified by affinity chromatography on a RGDW column, has been characterized previously [23].

Human platelet and CHO extracts were provided by Dr. Christiane Marie (UMR CNRS-UJF 5538, IAB, Grenoble) and purified αIIbβ3 complex purchased from Hemeris (Grenoble).

Synthetic peptides D-Gly-Arg-Gly-Asp-Ser (GRGDS), D-Gly-Arg-Gly-Glu-Ser (GRGES) were kindly prepared by Dr. Arlaud (IBS).

2.3. Peptide experiments

Maize calluses were cultured in the absence or presence (0.25 mg/ml) of either GRGDS or GRGES pentapeptides. Samples were viewed and photographed with a Nikon Microphot-FXA microscope (Nikon Europe B.V.).

2.4. Immunofluorescence localization

Maize calluses were fixed under vacuum in 4% paraformaldehyde, 0.5% glutaraldehyde and 0.1% Triton X-100 in a 50 mM KPO_4 buffer (pH 7.2). Fixed tissues were dehydrated and washed with ethanol and xylene before being embedded in paraffin. Embedded tissues were sliced into serial 6 mm sections and attached to microscopic slides coated with 3-aminopropyl-triethoxysilane (Sigma) in acetone. Paraffin sections of maize calluses, permeabilized with 0.5% Triton X-100 for 5 min, were probed using a 1:100 dilution of anti-β1 and P23 rabbit polyclonal antibodies in TBS (20 mM Tris-HCl, pH 7.4, 150 mM NaCl) containing 0.1% bovine serum albumin (BSA) or, as a control, rabbit non-immune serum and incubation with secondary antibody only. All samples were then washed with TBS and incubated

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with a goat anti-(rabbit IgG)-fluorescein conjugate and viewed with a Nikon Microphot-FXA microscope.

2.5. Protein extraction, glycoprotein isolation and immunoblot analysis

Maize calluses were immersed in liquid nitrogen and ground. The resulting powder was resuspended in extraction buffer: TBS containing 1% Triton X-100, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μM leupeptin (Boehringer-Mannheim Biochemicals) and 1 μM pepstatin (ICN). Protein extracts were dialyzed and concentrated by centrifugation through a Centricon 30 membrane (Amicon, Grace SA). Glycoprotein isolation on concanavalin A Sepharose and immunoblot analyses were performed as described previously [23].

2.6. Competition assays and immunoprecipitation

Competition assays were performed with CS3 (dilution 1:500), first incubated overnight at 16°C with increasing amounts of maize glycoproteins or platelet proteins before being incubated to microtiter plates coated overnight at 4°C with 0.4 μg human platelets as described elsewhere [23].

Immunoprecipitation was performed by incubation of 400 μg maize glycoproteins (non-treated or treated with 5 mM EDTA to dissociate the complex) with CS3 for 2 h at 4°C. The antigen-antibody complexes were then treated as described previously [23] and the immunoprecipitate was analyzed by electrophoresis and immunoblotting.

3. Results

3.1. Effects of RGD on maize callus growth and immuno-cross-reactivity between animal integrins and proteins at the cell surface

After 48 h of incubation of maize calluses with 0.25 mg/ml of GRGDS peptides, major changes of culture aspect, as shown in Fig. 1A, were observed. Calluses grown in the presence of GRGDS were compact nodulated embryogenic calluses with a large number of embryoids, whereas calluses grown in the presence of GRGES peptides (Fig. 1B) were indistinguishable from the control culture, being non-embryogenic.

Using the anti-β1 and anti-αIIbβ3 (P23) polyclonal antibodies and a FITC-conjugate secondary antibody, we examined maize callus cells. Judging by the localization of fluorescence under a conventional epifluorescence microscope, both anti-β1 (Fig. 2A) and P23 (Fig. 2B) integrin antibodies recognize antigens present on the surface of maize cells. In contrast, no fluorescence was observed in the absence of the primary antibody (Fig. 2C) or with the pre-immune rabbit antiserum alone (Fig. 2D).

3.2. Glycoproteins in maize cross-react with antibodies to human integrins

In order to determine whether maize callus cells contain molecules immunologically related to animal integrins immunoblotting experiments were performed. Glycoproteins were isolated using a concanavalin A Sepharose affinity column and fractions eluted with α-methyl-D-mannose were analyzed on SDS-PAGE gels, transferred to nitrocellulose and probed with the polyclonal antibodies anti-β1 and anti-αIIbβ3. Under reduced conditions the anti-β1 antibody immunodetected one polypeptide of 120 kDa (Fig. 3, lane 2), slightly smaller than the polypeptide (130 kDa) detected in Chinese hamster ovary (CHO) extracts (Fig. 3, lane 1) and the anti-αIIbβ3 antibody P23 recognized two bands of 30 and 60 kDa (Fig. 4, lane 2). The apparent molecular masses of the polypeptides immunodetected in human platelet extracts are in good agreement with those previously published [26]. The platelet glycoprotein is a calcium-dependent heterodimer composed of two distinct subunits. αIIb (GPIIb) is composed of two disulfide-linked polypeptides consisting of a heavy (GPIIbα) and a light chain (GPIIbβ) with estimated molecular masses of 116 and 25 kDa, respectively (Fig. 4, lane 1). β3 (GPIIIa) is a single chain polypeptide which has an apparent molecular mass of 100 kDa when reduced (Fig. 4, lane 1). Only the 60 kDa band is immunodetected with two different anti-β3 antibodies: CD61 (Fig. 4, lane 3) and Ly7 (Fig. 4, lane 4) or ZACA raised against the αIIb heavy chain (Fig. 4, lane 5) suggesting the presence in maize of two polypeptides with similar apparent molecular masses which are related to the β3 and αIIb heavy chain (GPIIbα) polypeptides, respectively. In addition, comparison of the bands detected with P23 (60 and 30 kDa) and ZACA (only the 30 kDa) suggests that the 30 kDa band is related to the αIIb light chain GPIIbβ.

3.3. A maize glycoprotein and the human platelet glycoprotein αIIbβ3 have similar structural domains

To characterize the structural relatedness between the maize proteins immunodetected with P23 and platelet αIIbβ3 receptor, antibody competition assays and immunoprecipitation experiments were performed. Data shown in Fig. 5 illustrate the competition observed for CS3, an anti-αIIbβ3 complex antibody, with maize glycoproteins and as a control platelet proteins. The competition observed with plant glycoproteins is similar to that observed in the homologous competition showing that the plant protein shares an immunologically related domain with its animal counterpart.

Immunoprecipitation experiments were performed to further analyze the structural properties of the plant complex.

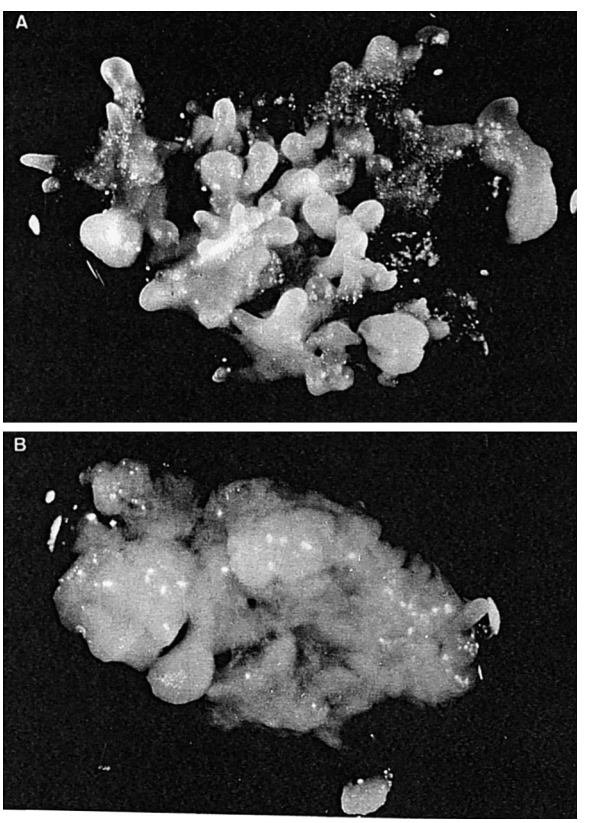

Fig. 1. Effect of the GRGDS (A) and GRGES (B) peptides in maize callus growth.

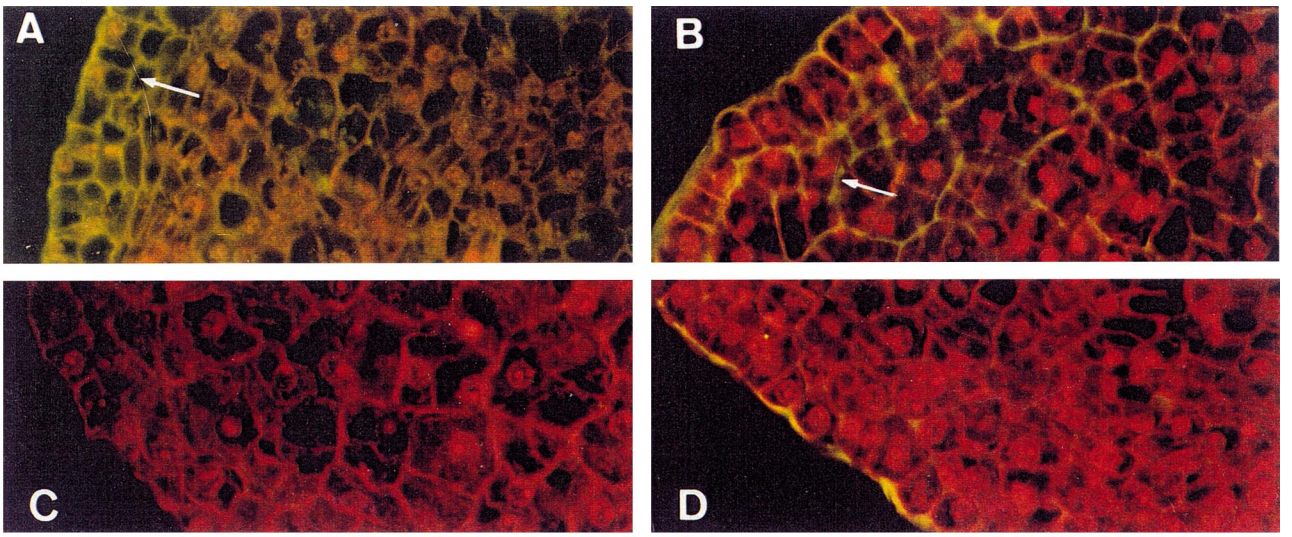

Fig. 2. Indirect immunofluorescent localization of integrin-like proteins at the surface of frozen sections of maize calluses. Sections were probed with the P23 (A) and anti-β1 (B) antibodies. Controls with secondary antibody alone (C) or rabbit non-immune serum (D) show no localization. Arrows in A and B indicate the cell surface. Magnification ×100.

Maize glycoproteins, non-treated or treated with 5 mM EDTA to chelate the divalent cations and dissociate the complex, were immunoprecipitated with CS3. Immunoprecipitates were separated under reducing conditions and blots were probed with P23. The results show that mAb CS3 only immunoprecipitated the complexed form of the protein with two bands at 60 kDa and 30 kDa (Fig. 6, lane 1) since no polypeptide is detected after dissociation of the complex with EDTA (Fig. 6, lane 2). No band was observed after immunoprecipitation using protein G alone (data not shown).

3.4. *A maize protein cross-reacts with AcAt2, a polyclonal antibody raised against Arabidopsis thaliana proteins purified by RGD peptide affinity*

Under non-reduced conditions both AcAt2 and P23 recognize one band in maize extracts with an apparent molecular mass of 90 kDa (Fig. 7, lanes 1 and 2, respectively), whereas two bands cross-react with P23 under the same non-reduced conditions with platelet extracts (Fig. 7, lane 3). As expected, under non-reducing conditions both β3 and αIIb show characteristic shifts in electrophoretic mobility: β3, due to its high cysteine content, migrates at 90 kDa and αIIb in its disulfide-linked form at 130 kDa.

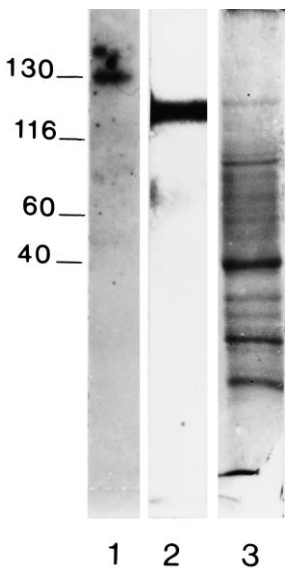

Fig. 3. Reactivity of the anti-β1 antibody with CHO extracts (lane 1) and 40 μg of maize glycoproteins (lane 2) separated under reduced conditions. Coomassie brilliant blue staining of maize glycoproteins (lane 3).

4. Discussion

We present evidence for the effect of RGD synthetic peptides on maize callus growth and the presence on the cell surface of proteins immuno-related to animal integrins. When maize calluses are grown in the presence of RGD peptides, enhanced embryogenesis is observed compared to maize calluses grown in the absence or presence of the control pep-

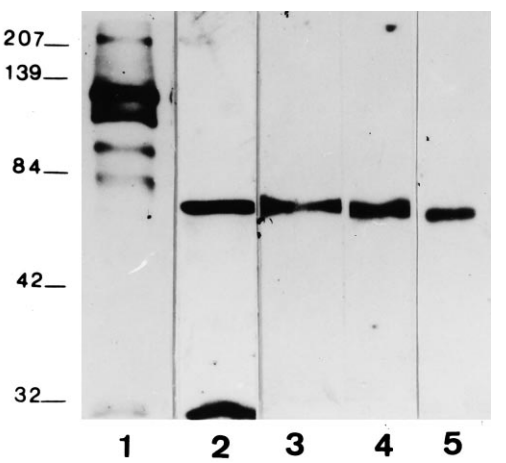

Fig. 4. Western blot of maize glycoproteins with different anti-αIIbβ3 polyclonal antibodies. 40 μg of maize glycoproteins were separated under reduced conditions and probed with P23 (lane 2), CD61 (lane 3), LY7 (lane 4) and ZACA (lane 5) antibodies. Purified αIIbβ3 human integrin (lane 1) was used as control with P23.

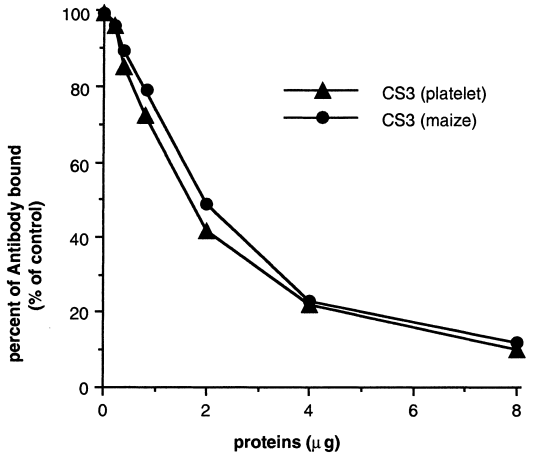

Fig. 5. Inhibition of the binding of CS3, a complex specific mAb to human platelet αIIbβ3 integrin, by maize glycoproteins and, as a control, platelet proteins. Increasing amounts of maize and platelet proteins were incubated with CS3 before being added to microtiter plates coated with 0.4 μg human platelet proteins. Detection of bound antibodies is expressed as percent of bound antibody in the absence of competitor. All experiments were done in triplicate.

tide RGE. In plants, the ability to form embryos is not restricted to germ cells. Generation of a totipotent state in somatic cells with competence for reinitiation of the whole developmental cycle through embryogenesis has been used in many biochemical and molecular studies to analyze early events in higher plant embryogenesis [27,28].

Excised plant tissue is first cultured in the presence of a high concentration of auxin which induces cell division and proliferation at the surface of a synthetic medium. In this heterogeneous cell proliferation only a limited number of cells exhibit embryogenic potential [29]. This fraction is highly variable among plant species and depends on the genotype and tissue origin of the primary explants [30,31]. Studies with a variety of lower plants seem to indicate that regeneration could be initiated by the mechanical or physiological isolation of cells from the integrative force of the multicellular tissue or organ. In these lower plants, such as multicellular algae or moss leaves, plasmolysis has been used to initiate regeneration and it is believed that plasmodesmata play an important role in integrative cell-cell communication [32].

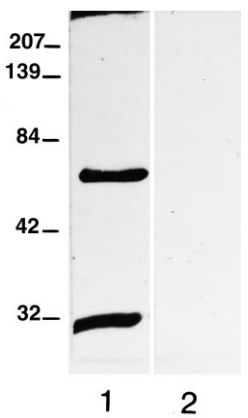

Fig. 6. Immunoblots of integrin-related proteins after immunoprecipitation. Maize glycoproteins were incubated with CS3 in the absence (lane 1) or presence (lane 2) of 5 mM EDTA. Immunoprecipitates were separated under reduced conditions and probed with P23.

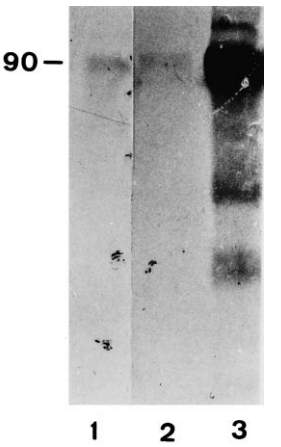

Fig. 7. Western blot of maize glycoproteins with AcAt2. Maize glycoproteins were separated under non-reducing conditions and probed with AcAt2 (lane 1) and P23 (lane 2). Purified αIIbβ3 human integrin (lane 3) was used as control with P23.

Plasmolysis also increases the frequency of regeneration and the degree of synchronous development in suspension cultures of wild carrot *Daucus carota* L. and the enhancement of embryogenesis was interpreted as an increase in the regeneration of cells which have become physiologically isolated from the tissue of origin [33]. We [23] and others [34] had previously shown that RGD-containing peptides induce aberrant cell wall-plasma membrane interactions when added to suspension cell medium. The effect observed with the RGD peptide on maize callus growth could therefore be a result, as discussed above, of the dissociation of the plasma membrane from the cell wall, allowing such cells to be free of the integrative force and to become embryonic.

In animals, most of the adhesive ligands to integrins bear the RGD recognition sequence and RGD peptides together with specific antibodies have been used to study the functions of integrin receptors. The involvement of ECM proteins such as fibronectin and vitronectin in the adhesion of cells to one another and to the ECM, in cell migration, in cytoskeletal organization and polarization of cells, in controlling cell proliferation and differentiation, and in affecting intracellular signalling processes and gene expression have all been documented [12,35].

Both immunological and functional data indicate that plants may use some of the same molecules to mediate plasma membrane-cell wall adhesion. A vitronectin-like protein has been detected in several species of plant: tomato, soybean, broad bean and lily [36,37]; in tobacco [38] and carrot suspension culture cells [39]; in the slime mold *Physarum polycephalum* [40] and the brown alga *Fucus distichus* [41,42]. Antibodies to human fibronectin stain the walls of salt-adapted tobacco cells, which are more adhesive than non-adapted cells [38]. In addition to the use of RGD peptides, the presence of integrin-like proteins in plants relies on immunoblot cross-reactivity with antisera to β3 [34,23] and β1 integrin subunits [41,43–45] as well as with the αIIb subunit [23].

Our results, with different antibodies raised against animal integrin subunits or plant proteins purified by affinity chromatography on a RGD column, indicate the presence of such related proteins in maize callus extracts. The 120 kDa band immunodetected with the anti-β1 antibody in maize is similar

in relative molecular mass to this β-integrin family in animals, whose relative molecular mass ranges between 95 and 130 kDa when separated under reducing conditions [46]. The 120 kDa band in maize calluses is also similar to the 120 kDa polypeptides detected with the same polyclonal antibody in onion [44], the fungus *Saprolegnia* [43] and the 84–116 kDa bands in *A. thaliana* [45], but is larger than the bands of 55 and 76 kDa in maize pollen and 92 kDa in *Fucus* zygotes [41].

In an earlier study with *Arabidopsis* and *Rubus* cells [23], using a combination of polyclonal and monoclonal antibodies, we have shown the presence of a surface protein sharing epitopes and structural properties with the human αIIbβ3 integrin. Here, using the same antibodies (with the notable exception that the P23 antibody was further purified by preabsorption with the αIIbβ3 integrin) we also present immunological and biochemical evidence for the presence in maize calluses of a related receptor. Under reduced conditions, the 60 and 30 kDa bands detected with P23 in immunoblot analysis with glycoproteins or after immunoprecipitation with CS3 are similar to the size reported for the *A. thaliana* polypeptides. The 60 kDa band which is detected with two different anti-β3 antibodies and an anti-αIIb heavy chain antibody suggests, as in *A. thaliana*, the presence of two co-migrating polypeptides related to the β3 and GPIIbα polypeptides, respectively. The structural conservation between the plant and animal receptor is further demonstrated with the 30 kDa band which is only detected with P23 but not with the anti-β3 and anti-GPIIbα antibodies and therefore could be related to the αIIb light chain subunit. As in animal, the αIIb heavy and light chains could be disulfide linked since under non-reduced condition, as shown in Fig. 7, the 30 kDa band is not detected with P23 and a shift to higher apparent molecular mass is observed.

AcAt2, a polyclonal antibody raised against *A. thaliana* proteins purified by affinity chromatography on a D-RGDW column and eluted with the RGD peptide, was shown to cross-react with the animal αIIbβ3 receptor and detected mainly a 90 kDa band under non-reduced conditions in *Arabidopsis* extracts [23]. Under the same conditions a band with similar molecular mass is also detected in maize callus extracts with both the plant and animal antibodies, indicating the presence of a related polypeptide in this plant. The cross-reactivity between proteins isolated from plants with antibodies raised against purified plant proteins and animal integrin represents an important step toward the characterization of such a receptor in plants. Furthermore, the fact that the plant antibody was raised against proteins purified by RGD peptide affinity indicates that the plant protein, like the animal counterpart, may interact with adhesive ligands via the recognition of the RGD tripeptide. In fact, it has been shown recently that high affinity RGD-binding sites are present at the plasma membrane of *A. thaliana* [47]. The effect observed with the RGD peptide on callus growth suggests that the plant receptor shares a common function with the animal integrin receptor which has been implicated in embryogenesis and cell differentiation.

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