AGAROSE-INDUCED EMBRYOID FORMATION : ACTIN MICROFILAMENTS AS DETERMINANTS OF THE MEMBRANE-MATRIX ADHESION SIGNAL TRANSDUCTION

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ABSTRACT

Agarose embedding of sunflower protoplasts induces a polarization leading to an asymmetric division pattern and embryoid formation. In this work we studied the role of actin microfilaments in transducing the adhesion signal. Freshly isolated protoplasts show cortical actin as punctate or short microfilaments. During the first 3 days of culture the cortical microfilaments become longer and organized in parrallel strands. The nuclei are surrounded by an actin basket extending towards the cortex by actin wires. This organization maintained untill the protoplast divides. The suppression of agarose-plasmalemma contacts by plasmolysing protoplast (12% mannitol) enhances the proportion of short microfilaments and decreases the parrallel network and the perinuclear cables of around 50%. Correlatively, the embryoid formation fall from 73% to 2.6%. Similarly, the inhibition of agarose-plasmalemma contacts by RGD peptide inhibit by 80% the embryoid formation. In this treatment the punctate microfilaments maintained up to 3 days and long cortical filaments are present in less than 30% of protoplasts; the perinuclear baskets formation is inhibited in 50% of the cells. Incorporation of phalloïdin in the culture medium, which induces polymerization of actin in long microfilaments, inhibits the embryoid formation in the same extent as RGD treatment; incorporation of cytochalasin, which hinders actin polymerization, inhibits the embryoid formation much more than RGD does.
These results indicate (i) that embryoid formation is triggered by RGD-dependent anchorage of the plasma membrane to the agarose matrix, (ii) this anchorage contributes to the actin cytoskeleton organization, (iii) the actin microfilaments integrity is necessary for the adhesion signal transduction.

Keywords: *Helianthus annuus*, protoplast, cytoskeleton, actin, microfilaments

INTRODUCTION

Embedding of plant cells or protoplasts in gelling medium is widely used to improve cell viability and plating efficiency. In *Helianthus annuus* (Chanabé et al. 1991), protoplast inclusion in a solid matrix induces changes in division pattern and development: protoplasts cultured in liquid medium divide symmetrically and form loose microcolonies, in contrast, when they are embedded in agarose, most of them divide asymmetrically and display a polar organization at the onset of their development giving rise to compact embryo-like structures (Petitprez et al., 1995). These embryoids progress to the heart-shape stage but do not develop further.

Recently, in a study of the cytoskeleton of agarose-embedded protoplasts, Caumont et al. (1997) reported that the agarose matrix interacts with cortical microtubules and stabilizes the cytoskeleton. In plant cells there is an increasing interest in transmembrane proteins because they could play structural and signaling roles in controlling cell polarity and morphogenesis. The synthetic tripeptide Arg-Gly-Asp (RGD), which inhibits the binding of integrin to vitronectin or fibronectin, inhibits plant cell growth (Schindler et al., 1989), membrane wall adhesions in *Pelvetia* (Henry et al., 1996) and polar axis fixation in *Fucus* (Shaw and Quatrano, 1996). These results suggest that adhesion sites could forward extra-cellular signals into the cell via the cytoskeleton. In our system, the agarose matrix could play the role of an artificial wall able to bind transmembrane proteins. In a previous work we shaw that membrane-agarose adhesion act on microtubules organisation (Barthou et al., 1999) In order to test if actin cytoskeleton is involved in such connections and adhesion signal transduction, we prevented protoplast adhesion to the agarose matrix by RGD peptides and examined modifications in actin filaments dynamics.

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METHODS

Plant material.

Sunflower hypocotyl protoplasts (Emil hybrid, Pioneer Hi Bred int.) were isolated according to Chanabé et al., 1989

Effect of RGD peptides.

Heptapeptides (stock solution 5mM) were kindly provided by R.F. Pont-Lezica (UPS-CNRS, Toulouse, Fr). In addition to the YGRGDSP sequence peptide, YGDGRSP was used as control in the same conditions.

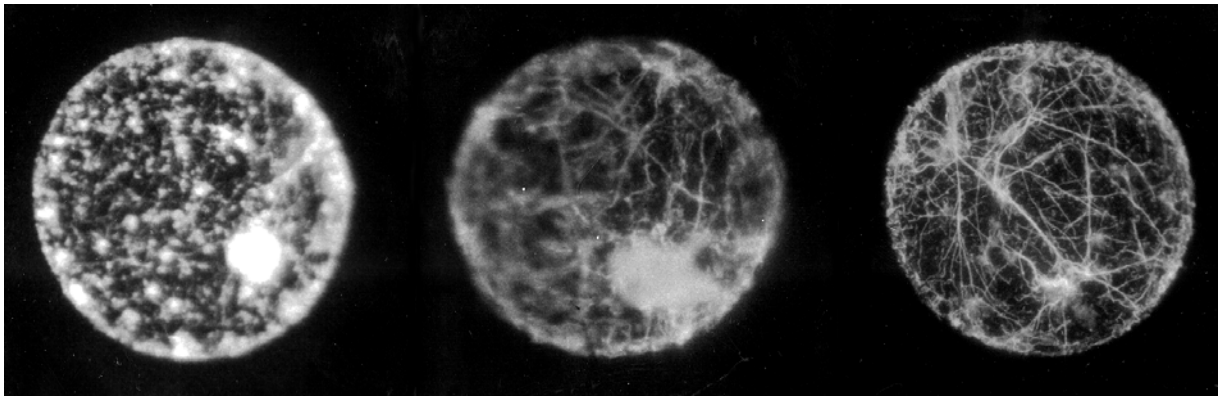
Immunocytolocalisation

Protoplasts were fixed for 1h in 2% paraformaldehyde containing 0.03% glutaraldehyde in microtubule stabilizing buffer, treated according to Caumont et al. (1997) and microtubules revealed by a mouse anti actin (Amersham)

RESULTS and DISCUSSION

Actin cytoskeleton dynamics

Freshly isolated protoplasts present mainly punctuated cortex with very few microfilaments: 45% of the protoplasts exhibit no labelling, 32%exhibit cortical dots (Fig.1A) and only 15% have long microfilaments (Fig.1B). After 1 day of culture in solid medium the punctuated cortex protoplasts decrease to 10% (Fig.2) whereas long microfilaments increase up to 35% of the protoplasts. At this time appear actin wires which connect a perinuclear basket to the cortical array of microfilaments (Fig.1C).This actin cytoskeleton organisation goes on during the first 3 days of culture, to reach a maximum at 5 days with 75% of the protoplasts having long cortical microfilaments and thick cytoplasmic wires linking the nuclear cage to the dens cortical array. At this time punctuated cortical labelling have disappeared.



A **B** **C**

Figure 1: Immunolocalisation of actin in agarose embedded protoplasts in standard conditions. The cortical actin appeared as dots in freshly isolated protoplasts (A), or long microfilaments (B). Deeper in the cytoplasm, long actin cables connect the nucleus (arrow) to the cortex (C)

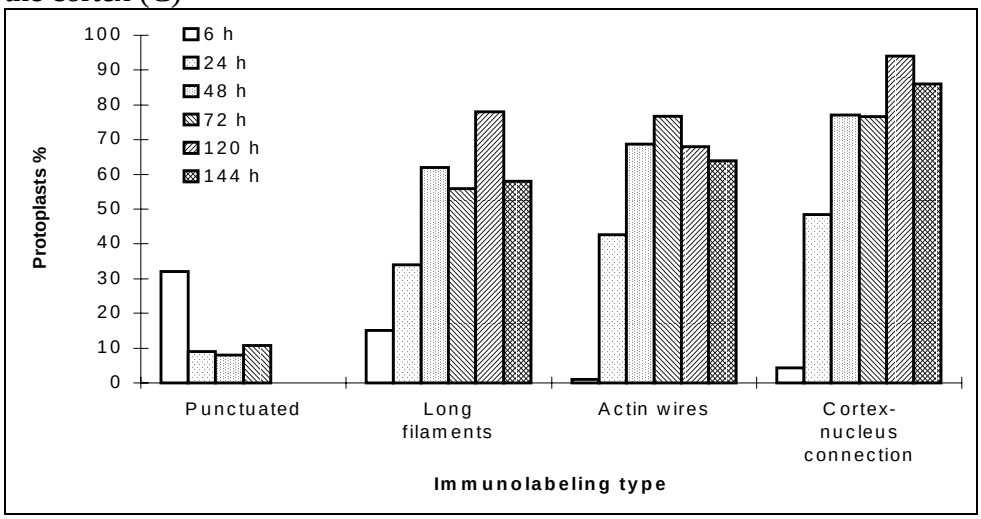


Figure 2: Actin cytoskeleton dynamics during the 6 first days of culture

Such a structure has already been describded in plant cells: two different actin filament systems in growing cell: cortical actin network covers the inner surface and an actin ensheathes the nucleus (Meindl et al., 1994). Basket shaped structures formed by F-actin in the nuclei of elongating protoplasts were reported by Kengen et al., (1993) and MFs of the nuclear basket following the contours of the nucleus extend outward in the cytoplasm to merge with the cables of the transvacuolar strands (Seagull et al., 1987).

RGD effects on actin cytoskeleton

In order to test the effect of membrane-agarose matrix adhesion on the actin cytoskeleton organisation, we applied 10µM RGD peptide in the culture medium. RGD treatment maintained short cortical microfilaments during the first 6 days , whereas in the DGR control they disappear at day 3. In the RGD treated protoplasts long cortical microfilaments set up more slowly and never in more than 35% of the cells (Fig.3A) whereas in the control this concern up to 75% of the protoplasts at day 5. Similarly the cytoplasmic actin wires and the connection between perinuclear basket to the cortex are markedly inhibited by the RGD treatment : 40% of the protoplasts at day 3 versus 75% in the control (Fig. 3B,C). Such a RGD peptide treatment did not significantly modifies the division rate of protoplasts, but significantly reduces the embryoid rate versus microcolonies differentiated after 21 days of culture (Barthou et al, 1999).

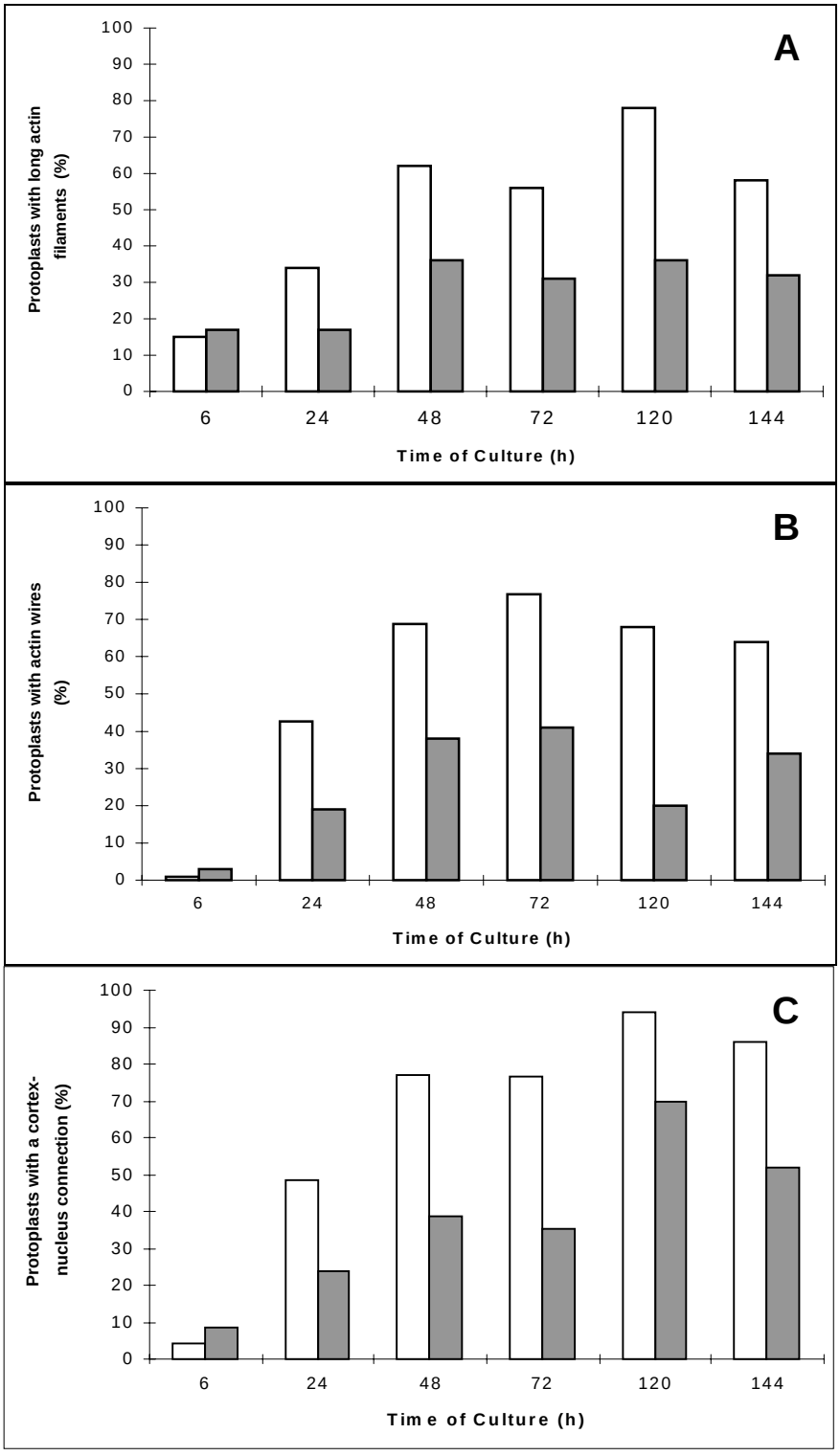


Figure 3 : RGD peptide effect on actin microfilaments organisation and dynamics

Breaking the continuum membrane-matrix by RGD peptide alters the actin structure. This is in agreement with previous reports on Mimosa protoplasts where Fleurat-Lessard et al.(1993) shawn that isolation of cells inducing rupture of connections with neighbouring cells alter the cytoskeletal organization. Similarly in mouth cells of Beroë the disassembly of cell junctions destroy the associated actin cytoskeleton (Tamm and Tamm, 1993). This could indicate that

interactions between the plasma membrane and the underlying actin cortex stabilise the membrane and affect the functions of the membrane proteins. (Hitt and Luna, 1994).

Actin is involved in transducing the adhesion signal

Adhesion of the protoplast to the agarose matrix seem to stabilise its actin cytoskeleton, and induces the embryoid differentiation. In order to test if the actin cytoskeleton could transduce the adhesion signal we applied phalloïdin, a microfilament stabilising drug, before or after RGD treatment. 10µM Phalloïdin treatment really stabilise actin microfilaments of sunflower protoplasts: it lowers non labelled, diffuse and punctuated labelling (Fig. 4) and increased protoplasts having long cortical microfilaments (60% versus 40%).

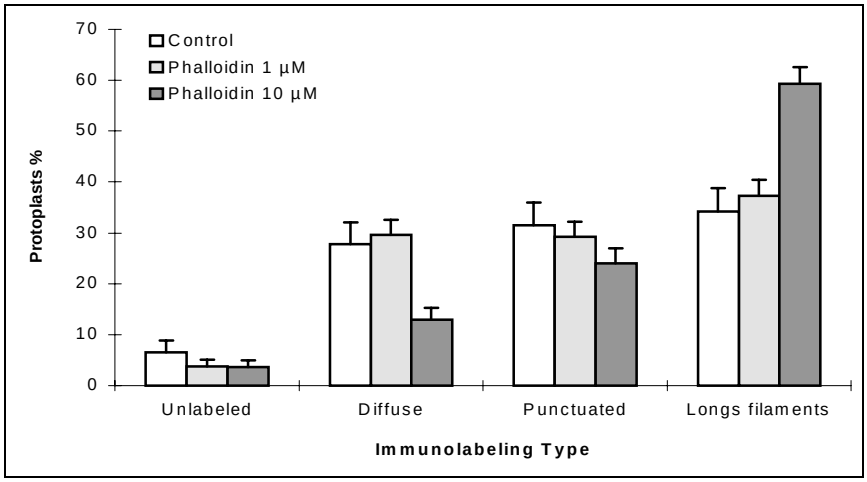


Figure 4: Effect of phalloïdin on actin labelling and organisation

Such a 10µM phalloïdin treatment did not modifies the division rates of protoplasts (Table 1) but when phalloïdin came after RGD it lowered its inhibiting effect on embryoid differentiation, in contrast when phalloïdin came before RGD it slightly widen the embryoid reduction. This suggest that actin cytoskeleton structure interact with adhesion/unadhesion sensing of the protoplast.

Table 1: Division rate, measured at day 8, and embryoid differentiated after 21 days of culture of protoplasts in agarose medium.

	DIVISION RATE (%)	**EMBRYOID (%)**
CONTROL	**56,86+-2,62**	**76,87+-3,64**
RGD	**61,93+-2,67**	**15,20+-3,21**
RGD / PHALOÏDIN	**60,95+-2,75**	**28,63+-3,00**
PHALOÏDIN / RGD	**60,57+-2,74**	**8,72+-2,15**

Actin cytoskeleton may mediate transcription regulation, thus, Glogauer et al.(1997) have shown that elongational stresses applied on collagen-coated beads bound to the cell surface actively promotes the assembly of actin stress fibers. This could be concerned in various stages of developmental process, including embryogenesis as suggested by correlations between transient localized accumulation of actin and setting up of oriented asymmetric divisions in CN blastomeres(Waddle et al., 1994).

These results indicate that embryoid formation is triggered by RGD-dependent anchorage of the plasma membrane to the agarose matrix, this anchorage contributes to the actin

cytoskeleton organization, the actin microfilaments integrity is necessary for the adhesion signal transduction.

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