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Binding of plasma membrane glycoproteins to the cytoskeleton during patching and capping is consistent with an entropy-enhancement model

Judith A. Shiozawa [1], John F. Brandts [1,2]* and Bruce S. Jacobson [1]

Departments of [1] Biochemistry and [2] Chemistry, University of Massachusetts, Amherst, MA (U.S.A.)

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Concentrations of concanavalin A that induced patching and capping of cell surface receptors on *Dictyostelium discoideum* also induce binding of the receptors to the cortical cytoskeleton, which was isolated by density-gradient centrifugation. The receptors were solubilized by deoxycholate, purified by affinity chromatography, and used to determine whether the receptors bound directly to the cytoskeletal protein, actin. As the concentration of actin was increased, many of the receptors became bound to purified filamentous rabbit muscle actin, even in the absence of concanavalin A. As in the ligation-induced binding of receptors to the cortical cytoskeleton in cells, concanavalin A induced much stronger binding of the purified receptors to filamentous actin. The results were consistent with a previously stated hypothesis that induction of receptor binding to the cytoskeleton during their patching and capping is driven by clustering the receptors, which reduces their translational entropy and by doing so enhances their avidity for the cytoskeleton.

Introduction

A large body of information has been gathered which indicates that patching, capping and subsequent endocytosis of many cell-surface receptors involves an interaction with the cytoskeleton (for reviews, see Refs. 1–10). The consensus is that the receptors are induced to bind to the cytoskeleton after they are clustered by an external multivalent ligand. Once bound, an actomyosin sliding filament mechanism similar to that in muscle cells forces the clusters into a cap which is then endocytosed.

The cellular slime mold *Dictyostelium discoideum* possesses a variety of cell-surface glycoproteins which are driven to bind to a detergent-resistant cytoskeleton during patching and capping by concanavalin A [11]. Many of the concanavalin A receptors are transmembrane proteins [12] and have been shown to be associated with the cytoskeletal protein, actin [13]. This is also true of membrane glycoproteins in platelets [14] and tumor cells [15], which are also induced to bind to the cytoskeleton by concanavalin A ligation [16–18]. It is as yet unclear how concanavalin A drives these proteins to bind to actin during the process of patching and capping.

A simple thermodynamic model has been recently proposed [18] to explain how the clustering of transmembrane receptors may result in an enormous enhancement of these receptors for the cytoskeleton. The model focuses upon the direct entropy enhancement which results from the clustering of receptors, which then acts thermodynamically to drive the subsequent interaction with the cytoskeleton. The mechanism is schematically illustrated in Fig. 1. It assumes (see Fig. 1A) that transmembrane receptors interact weakly with multiple sites on the cytoskeleton in the absence of clustering. Strong binding of free receptors to cytoskeletal sites is prevented by the two-dimensional translational entropy, i.e., two degrees of translational freedom are lost for each receptor which binds to the cytoskeleton. On the other hand, if the receptors are preclustered, e.g., by the binding of multivalent ligands to external sites on the receptors, then their translational entropy has already been quenched (see Fig. 1B). They may then bind to the cytoskeleton with essentially no further loss in translational entropy, i.e., only two

* Present address: Max Planck Institute for Biochemistry, D-8033 Martinsreid, F.R.G.

Correspondence: Bruce S. Jacobson, Department of Biochemistry, University of Massachusetts, Amherst, MA 01003, U.S.A.

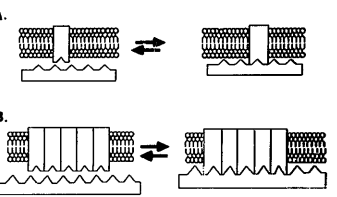


Fig. 1. Illustration of the entropy-enhancement model. (A) Binding of the free receptor to the cytoskeleton leads to the loss of two degrees of translational entropy for each interaction site that is satisfied. (B) Binding of the clustered receptor complex to the cytoskeleton leads to the loss of two degrees of translational entropy, but gives rise to many interaction sites.

degrees of translational freedom are lost for binding the entire receptor complex to the cytoskeleton. Thus, the clustered receptors act as a 'multivalent ligand' in terms of their two-dimensional interaction with the multivalent cytoskeleton, while the free unclustered receptors act as 'univalent ligands'. The multivalent advantage for binding ligands in three-dimensional systems, i.e., interactions in solution, is well known, and approximate calculations have shown that there will be analogous multivalent advantages in two-dimensional surface systems [18].

This simple model is able to make certain predictions about receptor–cytoskeletal interactions based on straightforward thermodynamic considerations about the process illustrated in Fig. 1.

(1) Receptors must have at least a weak interaction with cytoskeletal sites in the absence of clustering.
(2) The clustering of receptors which results from the binding of an external ligand, e.g., concanavalin A, will cause a marked increase in receptor binding to cytoskeletal sites.
(3) If transmembrane receptors can be removed from the membrane and solubilized in aqueous detergent solution, they should still show some tendency for enhanced interaction with the detergent-resistant cytoskeletal framework once they are crosslinked with the same external ligand.
(4) Interaction of crosslinked receptors with cytoskeletal sites should be weakened substantially if the cytoskeleton is dissociated into its 'subunits', since this also destroys the multivalent nature of the interaction and the entropy advantage which results therefrom.

We report here that the patching and capping of concanavalin A cell-surface receptors fits the predictions.

The above discussion does oversimplify a number of possible complications. Most importantly, it assumes that site–site interactions are equally strong when the clustered receptors are bound as when the free receptors are bound, so that only the differences in translational entropy become important. There may, for example, be a mismatch in the spacing of valencies so that the interaction sites on the receptor complex may be out of register with those on the cytoskeleton. Although these detailed geometric considerations are important, they may not be critical at the qualitative level discussed here, since, first of all, the cytoskeleton itself is probably fairly flexible and is likely to contain a large number of repetitive sites. In addition, the thermodynamic advantage in binding entropy which arises from receptor clustering is potentially enormous [18], so that some deterioration in site–site interaction energies or some completely vacant sites can probably be tolerated without affecting the overall picture.

Materials and Methods

Dictyostelium discoideum, strain AX-3 were grown in HL-5 medium [19]. Plasma membrane from vegetative cells were isolated as previously described in Ref. 20, except that the cells were lysed by nitrogen cavitation after equilibration at 400 lb/ln^2 for 10 min. The isolated membranes were solubilized in deoxycholate to a final concentration of 5% (w/v) detergent at 5 mg/ml protein [21] and centrifuged at $30000 \times g$ for 30 min. The supernatant was applied to a concanavalin A-agarose (Bio-Rad, Richmond, CA) column which measured 1.5×10 cm. Nonspecifically bound proteins were eluted from the column by washing with 0.5% sodium deoxycholate in 20 mM Tris-HCl (pH 7.6) followed by 0.2 M galactose in the same buffer. The concanavalin-A-binding fraction was eluted with 0.2 M methyl α-mannoside and dialyzed against 20 mM Tris-HCl (pH 7.6). The glycoproteins were used immediately or stored for short periods of time at −20 °C, since storage for longer than several weeks leads to spurious results. The glycoproteins were suspended to 100 μg/ml in 2% (v/v) Triton X-100 in 20 mM Tris-HCl (pH 7.6) and centrifuged at $160000 \times g$ for 1 h to remove any aggregates that might sediment with filamentous actin during the actin-binding studies.

The cortical or plasma-membrane cytoskeleton was isolated from concanavalin-A-treated and -untreated cells according to previous procedures (see Ref. 11). Rabbit muscle actin was isolated as previously described in Ref. 22 with additional purification by gel filtration on Sephadex G150 using the following monomeric actin buffer: 2 mM Tris-HCl/0.2 mM ATP/0.2 mM $CaCl_2$/0.5 mM dithioerythritol/0.02% NaN_3 (pH 8.0). When glycoprotein-actin binding studies were made, the actin in the above buffer was centrifuged at $160000 \times g$ for 1 h then used directly or after addition of KCl to 100 mM and $CaCl_2$ to 2 mM to form filamentous actin. For F-actin binding, the solution

containing the glycoproteins was also brought to 100 mM KCl and 2 mM $CaCl_2$ and centrifuged before use.

SDS-PAGE, detection of glycoproteins using HRP conjugated concanavalin A with 4-chloronaphthol as the chromogenic reagent, and protein determination were performed as previously described in Ref. 12. Detection of concanavalin-A-binding proteins in gels after electrophoresis with fluorescein-conjugated concanavalin A was done according to previous procedures described in Ref. 23.

Results

The glycoproteins that were affinity-purified by concanavalin A-Sepharose chromatography are shown in Fig. 2. Since the receptors were solubilized and chromatographed in the presence of the nondenaturing detergent, it was possible that other proteins might copurify with the concanavalin A receptors under the mild conditions used for the purification. To ensure that this had not happened, the purified concanavalin A receptors were examined very carefully for possible contamination by other proteins that were not concanavalin-A-binding proteins. Under the strongly denaturing conditions used in SDS-PAGE, any protein–receptor interactions that might lead to copurification in the Sepharose procedure should be disrupted and every protein should migrate independently. The results in Fig. 2 show two identical electrophoresis gels of the affinity-purified receptors; one gel of proteins having been stained by general ^{125}I-labelling and the other having been stained by the specific HRP-concanavalin A method. A careful comparison shows that there are no additional bands that appear in the gel stained by the ^{125}I method, except for concanavalin A which bled off the column. Therefore, it seems likely that the purified concanavalin A receptor preparation contains only proteins which themselves bind to concanavalin A. Since there was some variability in the qualitative com-

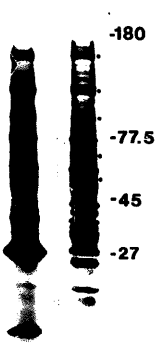


Fig. 2. Comparison of the HRP-conjugated-concanavalin A staining pattern with the ^{125}I-labeled protein pattern of affinity purified, cell-surface concanavalin A receptors. Left lane, autoradiogram of ^{125}I-labeled proteins and right lane, HRP-conjugated-concanavalin A staining for glycoproteins (GP). Glycoproteins eluted from the concanavalin A affinity column were dialyzed against 10 mM Tris-HCl (pH 7.6) labeled with ^{125}I by the chloramine-T procedure, electrophoresed by SDS-PAGE, electroblotted, stained with HRP-conjugated-concanavalin A and exposed to X-ray film as indicated in Materials and Methods. The positions of the molecular mass standards are indicated on the right side in kDa. Dots represent the major concanavalin-A-binding proteins consistently found in all experiments.

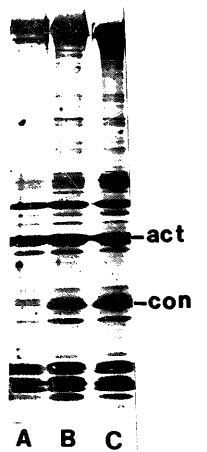
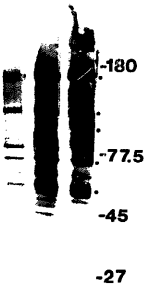


Fig. 3. Cortical cytoskeletons isolated from *Dictyostelium discoideum* after patching and capping with concanavalin A. Coomassie blue staining pattern of electrophorogram of proteins from cortical cytoskeletal preparations isolated from cells before (lane A) and after incubation with 20 μg/ml concanavalin A for 5 min (lane B) or 15 min (lane C). Cells were patched after 5 min and capped after 15 min (see Ref. 11). Protein load in each lane was 25 μg. Position of actin is indicated by 'act' and concanavalin A by 'con'.

position of the concanavalin-A-binding proteins in the different types of experiment carried out dots have been used in the figures to indicate the major concanavalin-A-binding proteins that were consistently found in all experiments.

The cortical cytoskeleton was isolated from cells either not treated with concanavalin A or treated for various length of time to induce the formation of concanavalin A patches or caps. Little differences in the relative amounts of actin and two high-molecular-weight proteins (possibly myosin and a filamin-like protein see Ref. 24) were found in the isolates, regardless of whether the cells were not treated with concanavalin A (Fig. 3, lane A) or were patched (lane B) or capped (lanes C) with concanavalin A. On the other hand, the relative amounts of concanavalin A cell-surface receptors co-isolated with the cortical cytoskeleton was markedly effected by patching or capping with concanavalin A. Only one concanavalin-A-binding protein was found to



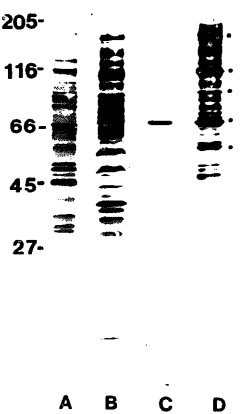


Fig. 4. Electropherogram of concanavalin-A binding proteins from various fractions of *Dictyostelium discoideum*. Lane A, whole cells; B, plasma membranes; C, cortical cytoskeletons before and D, after incubation with 20 μg/ml concanavalin A for 5 min at 21°C to patch the concanavalin A receptors. Concanavalin-A-binding proteins were detected on cellulose nitrate sheet electroblots of the electrophoretic gel using HRP-conjugated-concanavalin A as the detecting ligand [12]. Protein load was 20 μg per lane. Positions of molecular mass standards are indicated on the left in kDa. Dots indicate concanavalin-A-binding proteins consistently found in all cell and subcellular fractions.

Fig. 5. Cosedimentation of affinity-purified, cell-surface concanavalin-A-binding proteins with various concentrations of F-actin. Concanavalin-A-binding proteins (50 μg/ml) were mixed with in lane A, 50 μg/ml; B, 200 μg/ml; and C, 800 μg/ml of F-actin. After 30 min incubation, the mixtures (400 μl each) were centrifuged for 1 h at 130000 × *g* to pellet filamentous actin with bound glycoproteins. All solutions contained 1% Triton X-100/0.2 M methyl α-mannoside/100 mM KCl/2 mM $CaCl_2$. The concanavalin-A-binding proteins in the pellets were resolved by electrophoresis, electroblotted to cellulose nitrate sheets and were detected with HRP-conjugated-concanavalin A [12]. The positions of the molecular mass standards are indicated on the right in kDa. The clear area on the blot at 45 kDa is actin. The dots indicate concanavalin-A-binding proteins consistently found in all cell and subcellular fractions.

be associated with the cortical cytoskeleton in untreated cells (Fig. 4, lane C), whereas many glycoproteins were isolated with the cytoskeleton if the cells were first patched or capped with concanavalin A (Fig. 4, lane D). The concanavalin-A-binding proteins in the latter isolates were a subset of the total concanavalin-A-binding proteins in the cell or in the plasma membrane (Fig. 4, lanes A and B).

In order to test whether the concanavalin A cell-surface receptors bound directly to actin and whether concanavalin A ligation would increase the binding, the receptors were purified from plasma-membrane preparations by affinity chromatography on concanavalin A-Sepharose, subsequently mixed with purified rabbit muscle actin and then centrifuged to pellet filamentous actin with bound receptors. Since the concanavalin A receptors alone did not sediment, whether or not they were ligated by concanavalin A, it was assumed the

receptors in the actin pellets were there because they had bound to the actin filaments. The concanavalin-A-binding proteins in the pellets resolved by electrophoresis were detected either by staining of the gel with fluorescein-labeled concanavalin A [23] or by electroblotting to nitrocellulose sheets and subsequent detection with an enzyme-linked assay using HRP-conjugated concanavalin A and 4-chloronaphthol as the chromagen [12]. Both types of concanavalin A staining gave the same results. Since small amounts of concanavalin A bled off the column during the affinity purification of the receptors, methyl α-mannoside, at a concentration used to release to glycoproteins from the affinity column, was routinely used to inhibit concanavalin A ligation of the glycoproteins in solution. The methyl α-mannoside concentration appeared to be saturating, since doubling it did not change the results when these receptors bound to actin.

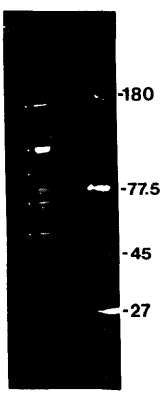


Fig. 6 Effect of concanavalin A ligation on receptor cosedimentation with actin. Affinity-purified, cell-surface concanavalin-A-binding proteins were incubated for 30 min with filamentous actin (lanes A and B) or globular actin (lanes C and D) in the presence of 0.2 M methyl α-mannoside to inhibit concanavalin A binding in lanes A and C. The concentration of actin was 50 μg/ml, concanavalin-A-binding proteins 50 μg/ml and concanavalin A 3 μg/ml. F-Actin with bound concanavalin-A-binding proteins was isolated as in Fig. 3. The concanavalin-A-binding proteins bound to actin were resolved by electrophoresis and detected by diffusion-staining of the gel with fluorescently labeled concanavalin A and using a long-wave ultraviolet light box for fluorophore excitation. The dots indicate concanavalin-A-binding proteins consistently found in all cell and subcellular preparations.

It was found that some concanavalin A receptors, in the presence of methyl α-mannoside to prevent concanavalin A ligation, became bound to filamentous actin as the F-actin concentration was increased (fig. 5, lanes A, B and C). This finding was consistent with the entropy-enhancement model [18], which postulates a weak intrinsic receptor–cytoskeleton interaction in the absence of ligation, the equilibrium of which could be shifted in favor of receptor binding if the concentration of F-actin were raised sufficiently. It was also found that addition of concanavalin A markedly increased the binding of the purified receptors to F-actin (compare lanes A and B in Fig. 6), while no effect was seen in the globular actin (lanes C and D: control experiment. Earlier approximate calculations using the present model indicated that clustering receptors by ligation may increase the affinity of the receptors for the cytoskeleton by many orders of magnitude [18].

The glycoproteins that bound to the F-actin were a subset of those present in the starting mixture (see Fig. 2), consistent with specific binding. If the binding had been nonspecific, one might have expected that the glycoproteins that were bound would have been more representative of those present in the starting mixture. In addition, high concentrations of BSA (1 mg/ml) did not interfere with the binding of plasma-membrane glycoproteins to cytoskeleton prepared by Triton/X-100 detergent extraction of whole cells, and, when specific binding of concanavalin A was inhibited by methyl α-mannoside, it was not enriched in the F-actin pellets as would be expected if it bound nonspecifically to actin (data not shown).

Discussion

Results from the present experiments are in accord with the qualitative predictions of the entropy-enhancement model [18] as enumerated in the introduction. Concanavalin A cell-surface receptors in *Dictyostelium discoideum* could be identified by specific staining. It was shown that one of these receptors exhibited significant binding to the detergent-resistant cortical cytoskeleton in the absence of concanavalin A. However, addition of concanavalin A to levels which induced patching on the cellular membrane resulted in the strong binding of a whole family of concanavalin A receptors to the cortical cytoskeleton (see Fig. 4).

Solubilized concanavalin A receptors were isolated by affinity chromatography in the presence of deoxycholate and the interaction of these receptors with rabbit muscle actin studied. Utilizing this model system of filamentous actin and solubilized concanavalin A receptors, no interaction could be detected at low concentrations of receptors and F-actin in the absence of available concanavalin A. However, as the concentration of F-actin was increased over a ten-fold range,

there was measurable binding of the receptors to F-actin (see Fig. 5). In the presence of concanavalin A, the binding of concanavalin A receptors was very strong even at low concentrations of filamentous actin (see Fig. 6). These results show that the concanavalin A receptors react weakly with the cytoskeletal component in the absence of ligation and that the interaction is enhanced significantly in the presence of concanavalin A.

All models for transmembrane signalling focus on the interdependency of external and internal sites on the membrane surface. The entropy-enhancement model [18] assumes that binding of a multivalent ligand to external sites will induce clustering of transmembrane receptors, which in turn enhances binding of a multivalent species, such as the cytoskeleton, to the internal sites, since the latter process may then proceed with hardly any further loss of two-dimensional translational entropy. In this way, binding to the two sets of sites is a cooperative process, and the free energy for binding of the external ligand is used to drive the subsequent interaction with the cytoskeleton via receptor clustering. This type of linkage between sites is based on well-established thermodynamic principles (see Ref. 18), and could be involved in other types of signalling not necessarily involving the cytoskeleton. Its simplicity is appealing, since it requires neither the existence of a ubiquitous cluster-linking protein [25] nor the occurrence of a conformational change of a receptor on one side of a membrane as the result of binding a ligand on the opposite side.

Although it is encouraging that our experimental results on the *Dictyostelium discoideum* system are in accord with the predictions of the entropy-enhancement model [18], more critical tests are necessary to establish the model's validity. We took the approach reported here as a first approximation. Five fairly abundant glycoproteins were identified and found to bind to F-actin in a way that was consistent with the entropy-enhancement model (see the dots next to the bands in Fig. 2, 4, 5 and 6). It now becomes feasible to determine quantitatively the effects of concanavalin A ligation on the binding of F-actin to the individual glycoproteins in nondenaturing detergent solutions and after reconstitution in phospholipid vesicles. Prior attempts by us to develop monoclonal antibodies for this purpose using plasma membranes as the immunogen were unsuccessful. As others have shown, when the plasma membrane was used as the immunogen the vast majority of antibodies that were generated were crossreactive with several proteins [26,27]. This could have been due to the presence of the highly antigenic sugar moieties on the proteins which, when removed by glycosidases, facilitated the production of antibodies to individual *Dictyostelium discoideum* plasma-membrane proteins [27]. Using the glycoproteins as isolated herewith subsequent affinity purification on actin as the antigen even without glycosidases should help alleviate past difficulties and allow us to generate the antibodies necessary to explore more quantitatively the entropy-enhancement model of concanavalin A receptor–cytoskeletal interactions.

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