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Double label freeze-etch study of the relative topography of concanavalin A and wheat germ agglutinin receptors at the myoblast surface

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This report records for the first time double-label freeze-etch electron microscopy of cells in culture. On L6 rat myoblasts, receptors for wheat germ agglutinin and concanavalin A were found distributed together in irregular granular microclusters of cell surface material up to 60 nm in diameter. Simultaneous localization of two different receptor families to such small regions using colloidal gold and ferritin to differentiate between lectin markers proved difficult in our hands. We were able to achieve the desired result using native concanavalin A and ferritin-conjugated wheat germ agglutinin, whose shadowed diameters are measurably different.

Analysis of the arrangement, rearrangement, and functional interaction of receptors at the eucaryote cell surface continues to be a central problem in the fields of biochemistry and cell biology Indeed with the progress that has been made in isolation and analysis of individual biomolecules, lack of detailed information regarding their topography in the membrane is one of the factors most severely limiting further advances. We have experimented with deep etching of freeze-fractured preparations as a means of direct sample visualization for correlation with other physical data The technique of fracturing specimens at very low temperature is of course well known for its unique ability to demonstrate extensive regions of cell membrane hydrophobic interiors However, the subsequent etching step that exposes membrane surfaces has been utilized rarely except on erythrocytes [1–6] The latter situation results from technical difficulties involved in extending the approach to cultured cells and tissues We recently reported a method for applying freeze-etch electron microscopy to cells in culture [7] The cell line studied was the rat myoblast, whose differentiation and fusion to form myotubes is thought to be accompanied by significant interactions involving glycolipids and/or glycoproteins (Ref 8 and references therein) To our knowledge this represents the only application of the technique to monolayer cell cultures. We described characteristic raised irregular patches 20–60 nm in diameter occupying 50% of the cell surface These prominent glycocalyx features extend at least 10–20 nm above adjacent membrane regions and seem to represent the extracellular portions of clustered groups of glycoproteins We record here the identification relative to one another of two receptor families amongst the glycocalyx surface features described above

The topographic arrangement of receptors at the myoblast surface has been considered by several groups in past using lectins Sawyer and Akeson [9] recorded a pattern of diffuse fluorescence (uniform labelling) when they employed fluorescent-labelled wheat germ agglutinin or con-

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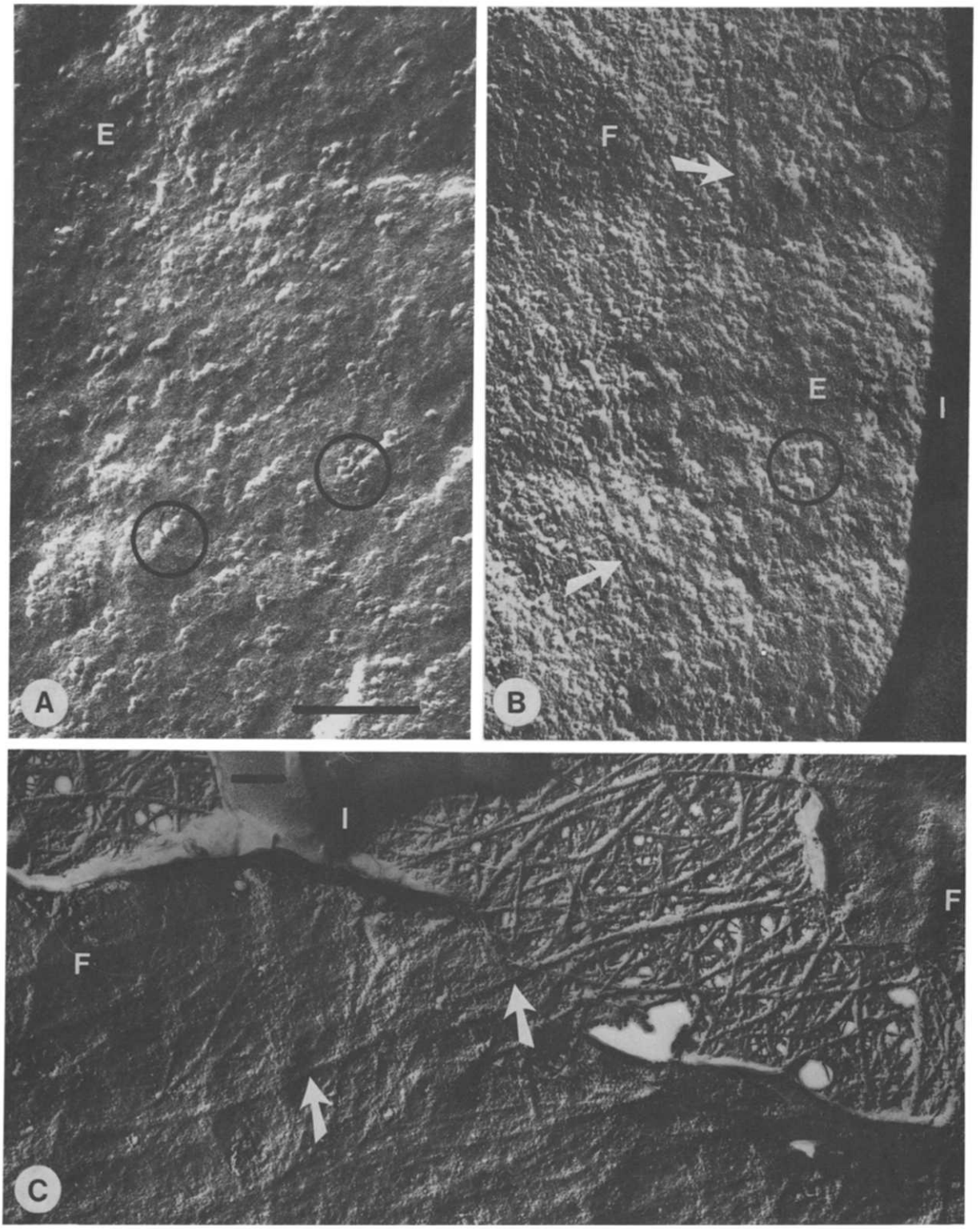
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canavalin A to mark the cell surface On the other hand Schlessinger et al [10] found that fluorescent photobleaching of labelled concanavalin A bound to the L6 myoblast surface gave correlation times for integral glycoproteins consistent with restricted motion, and have proposed as a basis for this the existence of glycoprotein clusters Fluorescence resonance energy transfer between labelled concanavalin A molecules at the myoblast surface has also been suggested to indicate the existence of glycoprotein receptor microclusters [11] Here we show that these previous considerations can be directly tested, and that receptors for both lectins are in fact organized into microclusters at the myoblast surface The two receptor families are topographically mingled, although apparently not totally homogeneously

Experimental details of the technique used have been given elsewhere [7], and are summarized in the caption to Fig 1 Fig 1A shows the upper surface appearance of myoblasts with bound ferritin-conjugated wheat germ agglutinin marking a family of receptors bearing terminal *N*-acetylglucosamine or *N*-acetylneuraminic acid [12] amongst the intrinsic glycocalyx surface features Ferritin appears as spherical particles 14 nm in diameter (uncorrected for shadow thickness) As we reported previously [7], the arrangement of these particles indicates localization of receptors for wheat germ agglutinin in diffusely-distributed microclusters associated with the raised glycocalyx prominences A typical surface region from a cell without added lectin appears for comparison in Fig 1B The cells shown were thoroughly prefixed with 2 5–4% glutaraldehyde prior to lectin addition, however, very similar results were obtained with unfixed cells incubated with the same lectin for up to 15 min Lectin binding was prevented by low concentrations of the inhibitory sugar, *N*-acetyl-D-glucosamine Note in Fig 1C that fibrils of the supporting collagen matrix deeply indent the lower membrane, consistent with the presence there of collagen binding proteins

Receptors marked in the myoblast by the tetravalent lectin, wheat germ agglutinin, are various glycoproteins and probably to a lesser extent glycosphingolipids [13] A very different family of glycoproteins, bearing terminal mannose, is marked by concanavalin A [8] We have taken the same ferritin-conjugated lectin approach to this latter group of receptors, and the typical result is shown in Fig. 2 Once again a diffusely distributed array of microclusters is picked out localized to glycocalyx prominences Sanwal and co-workers [8] have concluded on the basis of mutant selection, that a subset of the concanavalin A-binding glycoproteins is instrumental to the myoblast recognition and fusion process Note that in unfixed cells (Fig 2B) receptors for the tetravalent concanavalin A are significantly rearranged into patches after a 15 min incubation with lectin However even in this case the patch size is below the 200–400 nm resolution limit of light microscopy, hence using direct fluorescent staining techniques the pattern of lectin binding to unfixed cells would appear uniform and diffuse, as has indeed been recorded by others [9–11] Lectin binding was prevented by the presence of the appropriate inhibitory sugar (30 mM α-methyl-D-mannoside)

Fig 1 Distribution of receptors that bind wheat germ agglutinin L6 myoblasts were seeded into Linbro plate wells containing gold electron microscope grids coated with rat tail collagen They were grown to subconfluency on the grids in Alpha modified Eagle's medium with Earle's salts, supplemented with 10% horse serum Cells were washed twice by gently replacing the bathing medium with phosphate-buffered saline, and then fixed with fresh 2 5–4% glutaraldehyde for 60 min at 22° C prior to any manipulation or lectin addition After treatment, gold grids with cells attached were quenched, mounted in a Pfenninger device, and fractured as described by Pfenninger and Rinderer [14] Subsequent etching was for 10 min at −105° C prior to platinum shadowing (A) Prefixed myoblast incubated with ferritin-conjugated wheat germ agglutinin (0 75 mg/ml) for 15 min at 22° C A portion of an upper (outer) cell surface is shown at ×106400, and ferritin/lectin conjugate is seen as spherical particles 14 nm in diameter occurring in small clusters (examples circled) associated with glycocalyx prominences (B) Appearance of the upper surface of a similar prefixed cell without added lectin at the same magnification Irregular clumps of glycocalyx material up to 60 nm across (examples circled) protrude 10–20 nm from their surroundings, and the fracture face (membrane hydrophobic interior) displays typical intramembranous particles Arrows indicate the fracture face/etch face junction (C) Low magnification view (×42000) of the fractured lower membrane of a cell, demonstrating its close attachment to supporting fibrillar collagen matrix elements The latter are seen in the central right hand portion of 1C, and arrows mark the location of one such element beneath the fractured membrane I, ice, F, fracture face, E, etch face Shadow direction from bottom to top of page Bar is 200 nm

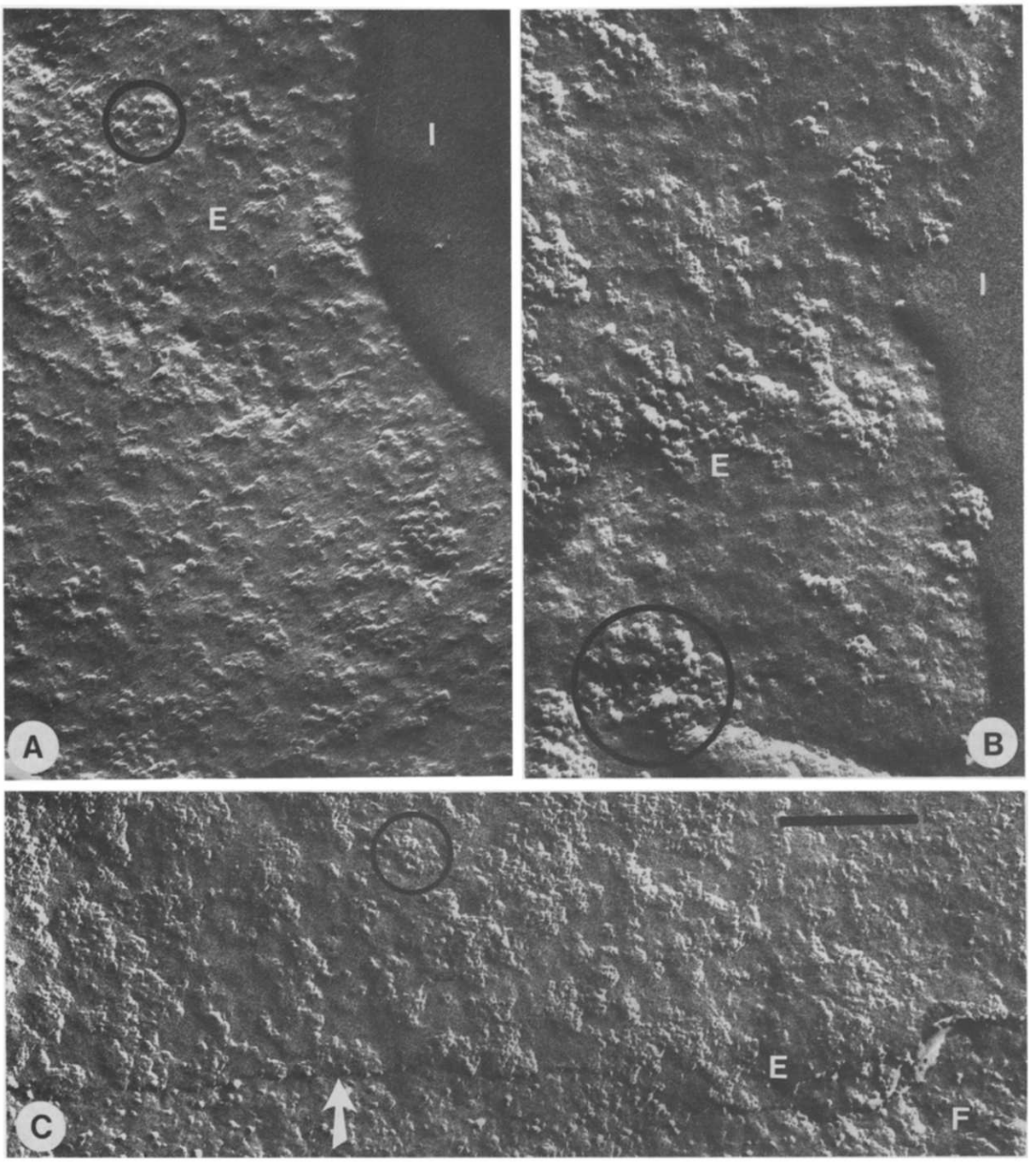

Fig 2 Distribution of receptors that bind concanavalin A (A) Prefixed cells were incubated with ferritin-conjugated concanavalin A (EY Laboratories, San Mateo, CA, 0 75 mg/ml) (B) Cells were fixed immediately after incubation with ferritin-conjugated concanavalin A (1 mg/ml) (C) Prefixed cells were incubated with native concanavalin A (0 5 mg/ml) In each case incubation time was 15 min at 22° C Ferritin particles are seen as precise spheres 14 nm in diameter (typical clustered groups circled in A, B) There is evidence of early patching in the unfixed cells Native lectin gives rise to granular deposits of 5–6 nm particles when shadowed with platinum (examples circled) Symbols and sample preparation as in Fig 1 Magnification ×106400 Bar is 200 nm

There remains the question of how the receptor family characterized by binding of wheat germ agglutinin is distributed relative to receptors for concanavalin A Certainly, as demonstrated above, they both follow the distribution of visible glycocalyx surface prominences We have found that native concanavalin A itself is recognizable as a spherical particle 5–6 nm in diameter (uncorrected for shadow thickness) against the cell surface glycocalyx features Yet it is smaller than ferritin hence it is possible to perform double label experiments in which the two populations of receptors are simultaneously labelled with native concanavalin A and ferritin-conjugated wheat germ agglutinin Fig 2C illustrates the appearance of native concanavalin A bound to the myoblast etch face It is not as well resolved as ferritin, and would be difficult to quantitate unambiguously against the intrinsic glycocalyx features However the distribution is the same as that seen for its ferritin-conjugated counterpart in Fig 2A

Results of double label experiments carried out under various conditions were examined to test the relative arrangement of the two receptor families Fig 3A shows the typical pattern obtained when both receptor families (in glutaraldehyde-

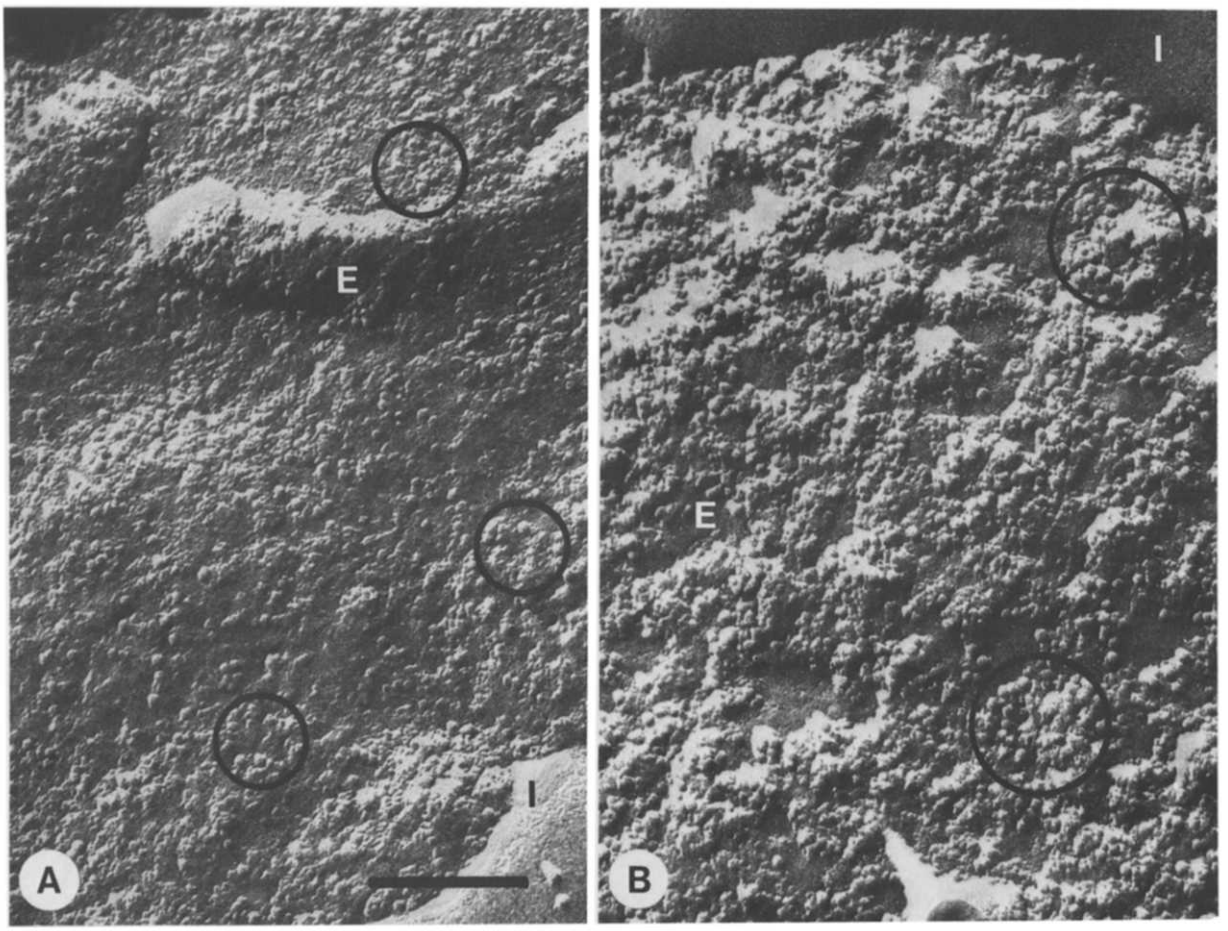

Fig 3 Myoblasts double labelled with concanavalin A and wheat germ agglutinin Prefixed (A) and unfixed (B) cells after 15 min simultaneous exposure at 22°C to native concanavalin A (0 125 mg/ml) plus ferritin-conjugated wheat germ agglutinin (0 75 mg/ml) Some receptor patching is present on the surface of cells fixed only after lectin incubation, and more lectin is bound in the latter case 14 nm ferritin particles and 5–6 nm concanavalin A particles occur in close proximity to one another (circled) but are not uniformly intermingled Symbols and sample preparation as in Fig 1 Magnification ×106400 Bar is 200 nm

fixed cells) are labelled simultaneously using native concanavalin A and ferritin-conjugated wheat germ agglutinin (Large) Ferritin-conjugated wheat germ agglutinin and (smaller) native concanavalin A occur together associated with the raised glycocalyx patches It is clear that their receptors occur in very close proximity to one another within the raised patches since the marker molecules are in direct contact It does seem though that the arrangement of wheat germ agglutinin receptors relative to concanavalin A receptors is not totally random the two lectins tend to exist in small groups rather than homogeneously mixed (which may reflect the presence of multiple binding sites on a given glycoprotein, grouped receptors, and/or positive cooperativity in the binding event) The same experiment performed on unfixed cells is shown in Fig 3B· there is considerably more lectin binding to unfixed cells, as we noted previously for wheat germ agglutinin in the absence of other lectins [7] The above general observations did not depend upon relative lectin concentration or the order of lectin addition

In order to simultaneously label two receptor families at the cell surface, it is necessary to employ two mutually distinct marker molecules Roth and Binder [15] solved this problem in a thin sectioning study of cultured epithelial cells by using one lectin covalently attached to colloidal gold and a second lectin conjugated to ferritin (peroxidase was also tested) These workers obtained highly satisfactory results, but did note that steric interference between the gold/lectin and ferritin/lectin markers at the spatially constrained cell surface could be a major limitation to marker binding and hence to the result obtained It has been demonstrated that, even in single label experiments, colloidal gold conjugation can restrict lectin access to surface receptors, especially when using larger gold particles [16] Colloidal gold has not been reported previously in double-label freeze-etching, although freeze-etch micrographs of colloidal gold-*helix pomatia* lectin bound to erythrocytes have been published [17] Use of native lectins in double label experiments has certain potential advantages over use of conjugated derivatives Firstly there is the stability, convenience, and unperturbed nature of the native species (the latter permitting direct comparison to other types of investigation with the same lectin) Perhaps more importantly, the smaller size of lectins without attached markers should permit labelling of receptors with minimal steric interference Unfortunately, identification of a given surface shadow in a tight cluster as concanavalin A vs ferritin-wheat germ agglutinin is often difficult Hence we have experimented with the ferritin-labelled lectins of Figs 1, 2, in various combinations and orders of addition [6,17], with concanavalin A-colloidal gold and wheat germ agglutinin-colloidal gold (both from Polysciences, Warrington, PA) In our hands this was less satisfactory than the approach demonstrated in Fig 3 for simultaneous freeze-etch identification of receptor families in small surface patches For one thing the technique of freeze-etch replica cleaning removes much of the colloidal gold from the replica [6], so that one is left with the problem of visually differentiating 14 nm ferritin shadows from 20 nm colloidal gold shadows This problem was not significantly easier in particle clusters than that of differentiating 6 nm concanavalin A from 14 nm ferritin shadows Furthermore the problem was compounded by the fact that even at the highest lectin-conjugate concentrations possible (and 2–3 h incubation times) we were unable to obtain the high simultaneous membrane densities of bound lectin that were easily obtainable with native lectin, and high density was necessary in deciding whether a given granular patch of surface material possessed both receptor families The overall impression was consistent with the results obtained using wheat germ agglutinin-ferritin and native concanavalin A

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