Dynamic membrane–cytoskeletal interactions: Specific association of integrin and talin arises *in vivo* after phorbol ester treatment of peripheral blood lymphocytes

(transmembrane interactions/tumor promoters/protein kinase C)

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ABSTRACT Members of the family of transmembrane integral membrane proteins called integrins have been implicated in forming attachments to actin microfilaments of the cytoskeleton. These attachments are thought to involve one or more intervening peripheral membrane proteins linked to integrin. To detect such possible linkages *in vivo*, the integrin molecules on the surfaces of intact chicken peripheral blood lymphocytes were collected into caps by cross-linking with specific antibodies, and the capped cells were examined by double immunofluorescence to determine whether particular cytoskeletal proteins were co-collected with the integrin. With resting lymphocytes, the capping of integrin did not result in any detectable redistribution of either talin, vinculin, or α -actinin inside the cells. However, if the capping was carried out upon the addition of phorbol 12-myristate 13-acetate (PMA) to the cells, then talin, but not vinculin or α -actinin, was found associated with the integrin caps. PMA is known to activate protein kinase C. These results suggest that after, but not before, PMA stimulation of intact cells, talin becomes linked either directly or indirectly with integrin, reflecting the formation of a membrane–cytoskeletal association that is metabolically regulated.

Connections between the plasma membrane and the cytoskeleton play important roles in many cellular phenomena, including cell–cell interactions, cell motility, and signal transmission across membranes. The basis for understanding the molecular nature of membrane–cytoskeletal connections was provided by the recognition of the existence and properties of two distinct classes of membrane proteins, integral and peripheral (1). It was proposed that specific peripheral proteins in the cytoplasm bind to the exposed hydrophilic domains of particular membrane integral proteins in the formation of each type of membrane–cytoskeletal attachment (1–3). However, except for erythrocytes, the detailed molecular composition and structure of membrane–cytoskeletal connections in eukaryotic cells are not known.

The associations of membranes with the actin microfilaments of the cytoskeleton have received much study, and in recent years a considerable number of peripheral and integral proteins have been proposed to participate in the associations (for review, see ref. 4). In particular, a family of integral membrane proteins called integrins (for reviews, see refs. 5 and 6) have been implicated as providing cytoplasmic membrane sites for the linkage of membranes to microfilaments. Immunofluorescence microscopic experiments have shown a close correspondence in the cell-surface distribu-

tions of integrin with actin microfilaments lying under the membrane (7–9). In addition, the cytoskeletal proteins talin (10, 11), vinculin (12), and α -actinin (13) have been localized by immunocytochemical methods at various sites of attachment of actin microfilaments to membranes, and each has therefore been considered a potential linker molecule between the membrane and the microfilaments (for review, see ref. 4). Furthermore, it has been reported (14) that talin binds to integrin *in vitro*, suggesting that a talin–integrin interaction might constitute a peripheral protein–integral protein linkage involved in the attachment of membranes to microfilaments. However, the *in vitro* integrin–talin binding was quite weak, and its physiological significance was therefore uncertain. To study possible physiologically relevant associations of integrin, we have carried out the antibody-induced “capping” (15) of integrin on the surfaces of intact lymphocytes and then examined by double immunofluorescence microscopic labeling whether inside the cells specific cytoskeletal proteins were collected with the integrin caps, as would be expected for any proteins that were significantly associated with integrin. We report that the capping of integrin on the surfaces of resting peripheral chicken lymphocytes did not result in a detectable redistribution of any of the cytoskeletal proteins talin, vinculin, or α -actinin. However, if integrin capping was carried out immediately after the intact lymphocytes had been treated with the phorbol ester tumor promotor phorbol 12-myristate 13-acetate (PMA), then talin, but not vinculin or α -actinin, was found associated with the integrin caps. PMA is an activator of protein kinase C (16). We infer from these results that there is no significant association of either talin, vinculin, or α -actinin with integrin in resting lymphocytes, but that upon PMA stimulation biochemical changes occur that induce a specific direct or indirect interaction between talin and integrin that does not involve vinculin or α -actinin. A dynamic membrane–cytoskeletal association, one that is capable of being metabolically regulated, has thereby been revealed.

MATERIALS AND METHODS

Cells and Reagents. Viable lymphocytes were isolated from citrated fresh chicken blood by a standard procedure using Histopaque-1077 (Sigma). The mouse monoclonal antibodies (mAb) JG9 (17) and 30B6 (9), each directed to a different epitope on the β -chain of chicken integrin (18), were produced as described. Affinity-purified rabbit antibodies directed to chicken gizzard α -actinin (19), vinculin (12), and

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Abbreviations: PMA, phorbol 12-myristate 13-acetate; mAb, monoclonal antibody(ies).

talins (20), were used in the earlier studies cited. The secondary antibody reagents were rhodamine-conjugated F(ab')₂ fragments of goat anti-rabbit IgG and biotin-SP-conjugated F(ab')₂ fragments of goat anti-mouse IgG (Jackson ImmunoResearch, Avondale, PA), along with fluorescein-conjugated streptavidin (Amersham).

Capping and Fluorescent Labeling. The intact lymphocytes were incubated with either mAb JG9 or 30B6 for 45 min, washed, and then incubated with the biotin-conjugated F(ab')₂ of goat anti-mouse IgG. All of these operations were carried out at 4°C. After washing, and to induce capping of the antibody-tagged integrin, the cells were then shifted to 37°C with or without the addition of PMA (50 ng/ml; Sigma) and incubated for 30–45 min. About 10⁶ cells were then plated on poly-L-lysine-coated coverslips, fixed with 3% formaldehyde, and treated with fluorescein-conjugated-streptavidin for 45 min. After washing, the fixed cells were permeabilized by brief treatment with 0.15% Triton X-100 and immunolabeled with either the rabbit anti-talin, anti-vinculin, or anti- α -actinin antibodies followed by the rhodamine-conjugated F(ab')₂ of goat anti-rabbit IgG, each for 45 min. After washing, the coverslips were mounted in 90% (vol/vol) glycerol/10 mM Tris-HCl buffer, pH 7.4, and examined with a Zeiss photomicroscope III (19).

RESULTS

The addition of mAb JG9 to the peripheral blood lymphocytes followed by biotin-conjugated F(ab')₂ fragments of goat anti-mouse IgG, all at 4°C, resulted in a uniform surface labeling (data not shown). If the labeled cells were warmed to 37°C, within 30 min the JG9 antigen was generally collected into a cap (Fig. 1 *A, D*, and *G*). The cells were then fixed, permeabilized, and indirectly immunolabeled for one of the cytoskeletal proteins, talin (Fig. 1*B*), vinculin (Fig. 1*E*), or α -actinin (Fig. 1*H*). There was no detectable redistribution of any of these proteins inside the capped cells. If, however, PMA was added to the cells that had been surface labeled at 4°C, and the cells were warmed to 37°C in the presence of PMA to induce capping of the JG9 antigen (Fig. 2 *A, D, G*, and *J*), then talin (Fig. 2 *B* and *E*), but not vinculin (Fig. 2*H*) or α -actinin (Fig. 2*K*), was found to be codistributed with the JG9 antigen caps. The same results were obtained if mAb 30B6 was used in place of mAb JG9 (data not shown) as is consistent with the finding (18) that the two mAbs are directed to the same antigen (see *Discussion*).

To demonstrate the specificity of this PMA-induced association of talin with the JG9/30B6 antigen, mAb 21D6, directed to an unrelated antigen (9), was used in place of JG9

FIG. 1. Double indirect immunofluorescent labeling of chicken peripheral blood lymphocytes after antibody-induced capping of mAb JG9. Each cell is represented by three photographs in a row. The photographs on the left (*A, D*, and *G*) illustrate the cell-surface labeling for the capped antigen, which is recognized by the mAb JG9. The photographs in the middle (*B, E*, and *H*) display the intracellular labeling for the cytoskeletal proteins talin (*B*), vinculin (*E*), or α -actinin (*H*). The photographs on the right (*C, F*, and *I*) show the Nomarski images of the cells. These pictures of single cells represent the majority of the cells displaying antibody-induced clustering of mAb JG9 in these experiments. Note the uniform distribution of labeling for the three cytoskeletal proteins. (Bar = 5 μ m.)

FIG. 2. Double indirect immunofluorescent labeling of PMA-treated chicken peripheral blood lymphocytes after antibody-induced capping of mAb JG9. Each cell is represented by three photographs in a row. The photographs on the left (A, D, G, and J) illustrate the cell-surface labeling for the capped antigen, which is recognized by the mAb JG9. The photographs in the middle (B, E, H, and K) display the intracellular labeling for the cytoskeletal proteins talin (B and E), vinculin (H), or α -actinin (K). The photographs on the right (C, F, I, and L) show the corresponding Nomarski images. These pictures of single cells represent the majority of the cells displaying antibody-induced clustering of mAb JG9 in these experiments. Note the codistribution of talin, but not of vinculin or α -actinin, with mAb JG9 caps. (Bar = 5 μ m.)

in a set of experiments similar to those shown in Figs. 1 and 2. Capping of the 21D6 antigen in the absence (data not shown) or presence (Fig. 3A) of PMA did not induce any change in the distribution of talin (Fig. 3B), vinculin, or α -actinin (data not shown).

DISCUSSION

A major problem of membrane-cytoskeletal associations at present is the determination of which integral and peripheral membrane proteins are linked molecularly to one another

and under what circumstances they are so linked. We have examined whether the cell-surface protein integrin (5, 6), which has been implicated in forming transmembrane connections between the extracellular matrix and the cytoskeletal microfilaments, is linked to particular cytoskeletal proteins *in vivo*. For this purpose, we have used antibody-induced capping of integrin on intact lymphocytes and double immunofluorescence-labeling experiments to determine whether any specific cytoskeletal proteins are collected with the integrin caps. Such a collection would be expected for any cytoplasmic proteins that were directly or indirectly linked to integrin in a physiologically relevant manner. By

FIG. 3. Double indirect immunofluorescent labeling of PMA-treated chicken peripheral blood lymphocytes after antibody-induced capping of mAb 21D6. (A) Photograph shows the cell-surface labeling for the capped antigen, which is recognized by the mAb 21D6; (B) photograph illustrates the intracellular labeling for talin; (C) photograph shows the corresponding Nomarski image of the same cell. These pictures of a single cell represent the majority of the cells displaying antibody-induced clustering of mAb 21D6 in these experiments. Note the uniform distribution of labeling for talin. (Bar = 5 μ m.)

this means we have shown that integrin is not detectably linked to either talin, vinculin, or α -actinin* in intact resting lymphocytes, but that upon stimulation of the lymphocytes by the phorbol ester PMA, integrin rapidly becomes associated with talin, but not with vinculin or α -actinin, inside the cells. The integrin-talin association after PMA treatment occurs within the time required to effect the capping of integrin (20–30 min). In these experiments, we have therefore demonstrated a dynamic membrane-cytoskeletal association.

The integrins are a family of membrane proteins (5, 6), consisting of a homologous set of heterodimer transmembrane integral proteins, with different members of the set represented on the surfaces of different cell types. Each heterodimer is made up of one smaller β -subunit (generally 110–120 kDa) and noncovalently linked to it, one unrelated larger α -subunit (150–170 kDa). The mAbs JG9 (17) and 30B6 (9) that were used in our experiments are directed to two different epitopes on the same chicken integrin β -chain (18).

The proteins talin (10, 11), vinculin (12), and α -actinin (13) have each been proposed as a candidate molecule serving to link membranes to microfilaments. Our results, demonstrating a specific association of integrin and talin, after PMA stimulation, suggest that talin can specifically serve such a linking function in a reversible fashion. However, our data do not necessarily imply that integrin and talin become directly linked to one another; other proteins (but neither vinculin nor α -actinin) might intervene between them.

The long-term (≈ 24 hr) effects of PMA on lymphocytes have been studied (cf. ref. 21), but in the present work we are concerned with short-term (≈ 30 min) effects. PMA is one of a group of tumor promoters that activates protein kinase C, apparently by binding directly to the enzyme (for review, see ref. 16). Phosphorylation of serine and threonine residues of appropriate protein substrates is thereby stimulated. We therefore suggest that this rapid induction by PMA of the association of integrin and talin is likely to be mediated directly or indirectly by the action of protein kinase C. The simplest possibilities are that either integrin or talin or both are phosphorylated on serine or threonine residues upon stimulation of the intact cells with PMA and that such phosphorylation is required for their interaction. Talin has

been shown to be a substrate for protein kinase C *in vitro* (22), but so far this is of uncertain relevance *in vivo*.

The concept of a membrane-cytoskeletal association that is subject to metabolic regulation has important consequences in cell physiology. It is quite likely also involved in the neoplastic transformation of fibroblasts by Rous sarcoma virus (RSV). The RSV oncogene encodes the protein p60^{v-src}, which is a tyrosine kinase. In RSV-transformed cells, talin (23) and integrin (24) become phosphorylated on tyrosine residues; this is accompanied by a breakdown of membrane-microfilament attachments and a rounding up of the normally flat adherent fibroblast. It is conceivable, therefore, that serine/threonine phosphorylation on the one hand, and tyrosine phosphorylation on the other, of talin and/or integrin have antagonistic effects (cf. ref. 25) on talin-integrin association. These biochemical mechanisms could therefore provide a means for on/off control of membrane-microfilament connections *in vivo*.

Horwitz *et al.* (14) have reported evidence for an interaction of integrin and talin *in vitro*. Although there was no binding of the two proteins detected in blotting experiments on gels or in sucrose gradients, equilibrium gel filtration experiments with gels permeated with talin indicated some binding. Under similar circumstances, vinculin did not show any binding to integrin. However, in mixtures containing vinculin, talin, and integrin, linkage of the three proteins was reported (14). In other *in vitro* experiments, evidence for an interaction of talin and vinculin was obtained (26). The relationship of these *in vitro* results to the *in vivo* findings reported here is unclear. If some kind of enzymatic modification is critical to the production of an interaction between integrin and talin, this would obviously affect the interpretation of any *in vitro* experiments that did not take such modification into account. It is possible, for example, that if the weak binding of integrin and talin observed *in vitro* was not simply artifactual, that it reflected an interaction of only the small fraction of the appropriately modified protein(s). Furthermore, in our *in vivo* PMA stimulation experiments, the association of talin with integrin caps was not accompanied by any detectable association of vinculin (Fig. 2), providing no evidence for a physiologically significant interaction of talin with vinculin such as has been reported to arise *in vitro* (14, 26).

As mentioned above, different homologues of the integrin family of protein complexes are present on the surfaces of different cell types (5, 6). For example, the glycoprotein IIb/IIIa complex on platelets (27) and the LFA-1 complex on human lymphocytes (28) have been shown to be related structurally to integrins. These proteins in their respective cells may therefore also exhibit a PMA-induced dynamic association with talin. We have indeed demonstrated this to

*In previous double immunofluorescence labeling studies from this laboratory (19), we found that α -actinin appeared to codistribute with the caps of several cell-surface antigens. We do not obtain similar results in the present study, however, and therefore suggest that the previous results may have been due to some residual cross-reaction between the affinity-purified and cross-absorbed secondary antibodies that were used.

be the case for LFA-1 on mouse T cells. The antibody-induced capping of LFA-1 resulted in codistribution of talin, but not of vinculin or α -actinin, on the intact T cells only after treatment with PMA (unpublished data).

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1. Singer, S. J. (1971) in *Structure and Function of Biological Membranes*, ed. Rothfield, L. I. (Academic, New York), pp. 145–222.
2. Singer, S. J. & Nicolson, G. L. (1972) *Science* **175**, 720–731.
3. Singer, S. J. (1974) *Annu. Rev. Biochem.* **43**, 805–833.
4. Singer, S. J., Maher, P. A., Rogalski, A. A., Kupfer, A. & Cox, G. F. (1986) in *Membrane Skeletons and Cytoskeletal-Membrane Associations*, eds. Bennett, V., Cohen, C. M., Lux, S. E. & Palek, J. (Liss, New York), pp. 261–268.
5. Buck, C. A. & Horwitz, A. F. (1987) *Annu. Rev. Cell Biol.* **3**, 179–205.
6. Hynes, R. O. (1987) *Cell* **48**, 549–554.
7. Chen, W.-T., Greve, J. M., Gottlieb, D. I. & Singer, S. J. (1985) *J. Histochem. Cytochem.* **33**, 576–586.
8. Damsky, C. H., Knudsen, K. A., Bradley, D., Buck, C. A. & Horwitz, A. F. (1985) *J. Cell Biol.* **100**, 1528–1539.
9. Rogalski, A. A. & Singer, S. J. (1985) *J. Cell Biol.* **101**, 785–801.
10. Burridge, K. & Connell, L. (1983) *J. Cell Biol.* **97**, 359–367.
11. Molony, L., McCaslin, D., Abernethy, J., Paschal, B. & Burridge, K. (1987) *J. Biol. Chem.* **262**, 7790–7795.
12. Geiger, B. (1979) *Cell* **18**, 193–205.
13. Chen, W.-T. & Singer, S. J. (1982) *J. Cell Biol.* **95**, 205–222.
14. Horwitz, A. F., Duggan, K., Buck, C. A., Beckerle, M. & Burridge, K. (1985) *Nature (London)* **320**, 531–533.
15. Schreiner, G. F. & Unanue, E. R. (1976) *Adv. Immunol.* **24**, 137–165.
16. Nishizuka, Y. (1986) *Science* **233**, 305–312.
17. Greve, J. M. & Gottlieb, D. I. (1982) *J. Cell Biochem.* **18**, 221–230.
18. Maher, P. A. & Singer, S. J. (1987) *Mol. Cell. Biol.*, in press.
19. Geiger, B. & Singer, S. J. (1979) *Cell* **16**, 213–222.
20. Kupfer, A., Singer, S. J. & Dennert, G. (1986) *J. Exp. Med.* **163**, 489–498.
21. Isakov, N., Scholz, W. & Altman, A. (1986) *Immunol. Today* **7**, 271–277.
22. Litchfield, D. W. & Ball, E. H. (1986) *Biochem. Biophys. Res. Commun.* **134**, 1276–1283.
23. Pasquale, E. B., Maher, P. A. & Singer, S. J. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 5507–5511.
24. Hirst, R., Horwitz, A., Buck, C. & Rohrschneider, L. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 6470–6474.
25. Cooper, J. A. & Hunter, T. (1983) *Curr. Top. Microbiol. Immunol.* **107**, 125–162.
26. Burridge, K. & Mangeat, P. (1984) *Nature (London)* **308**, 744–746.
27. Fitzgerald, L. A., Steiner, B., Rall, S. C., Lo, S. S. & Phillips, D. R. (1987) *J. Biol. Chem.* **262**, 3936–3939.
28. Kishimoto, T. K., O'Connor, K., Lee, A., Roberts, T. M. & Springer, T. A. (1987) *Cell* **48**, 681–690.