Dynamic Properties of Ankyrin in T Lymphocytes: Colocalization with Spectrin and Protein Kinase Cβ

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Abstract. Ankyrin is a well characterized membrane skeletal protein which has been implicated in the anchorage of specific integral membrane proteins to the spectrin-based membrane skeleton in a number of systems. In this study, the organization of ankyrin was examined in lymphocytes in relation to T cell function. Light and electron microscope immunolocalization studies revealed extensive heterogeneity in the subcellular distribution of ankyrin in murine tissue-derived lymphocytes. While ankyrin can be localized at the lymphocyte plasma membrane, it can also be accumulated at some distance from the cell periphery, in small patches or in a single discrete, nonmembrane-bound structure. Double immunofluorescence studies demonstrated that ankyrin colocalizes with spectrin and with the signal transducing molecule protein kinase Cβ (PKCβ) in tissue-derived lymphocytes, suggesting a functional association between these molecules in the lymphocyte cytoplasm. In addition, T lymphocyte activation-related signals and phorbol ester treatment, both of which lead to PKC activation, cause a rapid translocation of ankyrin, together with spectrin and PKCβ, to a single Triton X-100–insoluble aggregate in the cytoplasm. This finding suggests a mechanism for the reported appearance of PKC in the particulate fraction of cells after activation: activated lymphocyte PKCβ may interact with insoluble cytoskeletal elements like ankyrin and spectrin. Further evidence for a link between the subcellular organization of these proteins and PKC activity is provided by the observation that inhibitors of PKC activity cause their concomitant redistribution to the cell periphery. The dynamic nature of lymphocyte ankyrin and its ability to accumulate at sites distant from the plasma membrane are properties which may be unique to the lymphocyte form of the molecule. Its colocalization with PKCβ in the lymphocyte cytoplasm, together with its redistribution in response to physiological signals, suggests that structural protein(s) may play a role in signal transduction pathways in this cell type. Our data support the conclusion that ankyrin is not solely involved in anchorage of proteins at the plasma membrane in lymphoid cells.

ANKYRIN is a structural protein that was initially isolated from red cell membranes, where it links the cytoplasmic domain of the erythrocyte anion exchanger (band 3) to the spectrin-based membrane cytoskeleton. It is now known that erythrocyte ankyrin is a member of a family of closely related proteins that are found in many cell types (for reviews see Bennett and Lambert, 1991; Bennett, 1992). These proteins appear to play roles as adapters between specific integral membrane proteins and the spectrin skeleton. Cell surface proteins that have been shown to interact with ankyrin include the Na^+/K^+-ATPase and the amiloride-sensitive sodium channel in kidney, the voltage-dependent sodium channel in brain and at the neuromuscular junction, and the ankyrin-binding glycoproteins (ABGPs) in brain (for review see Bennett, 1992). Based on studies in various systems, it has been proposed that ankyrin functions either in the initial targeting of membrane proteins to specialized areas of the cell surface or in maintaining local concentrations of integral membrane proteins once the membrane domains have assembled (for example see Nelson and Hammerton, 1989; Bennett and Lambert, 1991).

The forms of ankyrin found in erythrocytes and brain are the best characterized members of the family. They consist of three independently folded domains: an 89–95-kD NH_2-terminal membrane binding domain, a 62-kD domain (with an apparent molecular mass of 72 kD on SDS gels) that binds to spectrin, and a 50–55-kD COOH-terminal regulatory domain. The 89–95-kD NH_2-terminal membrane binding domain of these ankyrins is almost entirely composed of 22 tandem repeats of 33 amino acids which are responsible, in the erythrocyte, for the high affinity interaction of ankyrin with the anion exchanger (Davis and Bennett, 1990). These

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repeats are of particular interest since they are closely related to 33-residue motifs found in a number of otherwise widely divergent proteins including yeast cell cycle control proteins, membrane proteins that regulate cell differentiation during development, and mammalian transcriptional regulators (for reviews see Michaely and Bennett, 1992; Nolan and Baltimore, 1992). It has been proposed that these repeats are involved in molecular recognition.

A 72-kD protein with similarities to erythrocyte ankyrin has been identified and characterized in a mouse T-lymphoma cell line (BW5147) (Bourguignon et al., 1986). Its ankyrin-like properties include the ability to bind purified erythrocyte spectrin, localization on the cytoplasmic side of the plasma membrane, and cross-reactivity with anti-erythrocyte and anti-brain ankyrin antibodies. In addition, it has a pI and a peptide mapping pattern similar to those of the 72-kD proteolytic fragment of erythrocyte ankyrin. This 72-kD ankyrin-like protein was found to be tightly associated with a major T lymphoma plasma membrane glycoprotein, GP85 (Pgp-1, CD44), with which it co-caps upon ligand-induced receptor cross-linking (Bourguignon et al., 1986; Kalomiris and Bourguignon, 1988). Based on these findings, and on the observation that lymphocyte spectrin (fodrin) is also localized at the cell periphery and co-caps with cross-linked surface molecules (Levine and Willard, 1983; Nelson et al., 1983; Bourguignon et al., 1985), it has been proposed that the 72-kD ankyrin-like protein is responsible for linking GP85 to spectrin and other cytoskeletal elements which are involved in lymphocyte cap formation (Bourguignon et al., 1986). While the physiological significance of the capping process is not understood, these findings suggest that lymphocyte ankyrin can interact with membrane components in a manner analogous to that observed in the erythrocyte and in various non-erythroid cells (see above).

Studies from our laboratory, however, focusing on the role of the cytoskeleton in the physiological response of tissue-derived lymphocytes to antigen-induced activation signals, have revealed an unexpected complexity in the subcellular organization of the ankyrin-binding protein spectrin in lymphoid tissues (Repasky et al., 1984; Pauly et al., 1986; Black et al., 1988). Lymphocyte spectrin is not invariably localized at the plasma membrane but can be found at several intracellular locations. Increasing evidence indicates that this heterogeneity reflects the dynamic nature of lymphocyte spectrin and its ability to redistribute within the cytoplasm in response to lymphocyte activation-related signals or other stimuli involved in lymphocyte maturation and/or function (Lee et al., 1988; Gregorio et al., 1992). Recent immunolocalization and biochemical analysis have revealed that an important component of the T cell receptor-activated signal transduction pathway, the enzyme protein kinase C (PKC)[1] βII, is associated (either directly or indirectly) with lymphocyte spectrin suggesting involvement of this cytoskeletal protein in signal transduction pathways. Since ankyrin is a spectrin-binding protein which is thought to mediate the interaction of cell surface molecules with the underlying membrane cytoskeleton (see above), we have examined its organization in lymphocytes exhibiting different patterns of spectrin distribution, particularly in those where spectrin is not associated with the plasma membrane. It was also of interest to determine the relationship of ankyrin to the redistribution of spectrin and PKCβ in response to activation-related signals.

In the present study, the subcellular organization of ankyrin was examined in untreated and activated lymphocytes to gain insight into its role in lymphocyte function. Ankyrin is shown to exhibit heterogeneous subcellular localization patterns, reflecting its dynamic responses to PKC activation-related signals. Our studies suggest that ankyrin and other membrane cytoskeletal components may carry out novel functions in lymphoid cells. The significance of our findings is discussed in terms of the reported properties of ankyrin in other cell types.

Materials and Methods

Cells

The T cell hybridoma, DO-11.10, was generously provided by Drs. P. Marrack and J. Kappler (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO) and grown as previously described (White et al., 1983). Tissue lymphocytes were isolated from lymph nodes taken from 2–3-mo-old female Balb/c mice. An enriched population of T lymphocytes was obtained by passage of lymph node cell suspensions over nylon wool (Julius et al., 1973). Purification was confirmed by immunofluorescence microscopy, using fluorescein isothiocyanate-conjugated anti-Thy 1.2 antibodies (ICN Biomedicals, Inc., Costa Mesa, CA) to identify T lymphocytes.

Antibodies

The anti-chicken erythrocyte ankyrin (goblin) antiserum used in this study was prepared by immunizing rabbits with antigen isolated from two-dimensional gels. The rabbit and goat anti-chicken erythrocyte α-spectrin antisera have been characterized previously (Black et al., 1988; Lee et al., 1988; Gregorio et al., 1992). The anti-PKCβ-specific monoclonal antibody (anti-PKCβ 1.3: GIBCO-BRL, Gaithersburg, MD) has been characterized extensively in our system (Gregorio et al., 1992). The pan-reactive anti-mouse α,β T cell receptor monoclonal antibody, H57-597 was generously provided by Dr. R. Kubo (Cytel, La Jolla, CA). Fluorescein and rhodamine-isothiocyanate conjugated antisera were purchased from ICN Biomedicals Inc. Horseradish peroxidase-conjugated antisera were purchased from Boehringer Mannheim Corp. (Indianapolis, IN) for immunoblot analysis and from ICN Biomedicals Inc. for electron microscopy.

Gel Electrophoresis and Western Blotting

For SDS-PAGE, chicken and mouse erythrocyte membranes were prepared as previously described (Granger et al., 1982; Bennett, 1983). Human erythrocyte ankyrin was isolated by the procedure of Bennett and Stenbuck (1980). For immunoblot analysis of ankyrin in T lymphocytes, conditions were optimized to reduce proteolysis (Bennett, 1983). Isolated cells were preincubated for 90 min in 10 mM diisopropylfluorophosphate (DIFP) in culture medium, and then washed with ice-cold PBS containing 5 mM Nα-p-tosyl-L-arginine methyl ester (TAME), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg ml^{-1} pepstatin A, 10 μM leupeptin, 1 mM EDTA, and 10 mM DIFP. Samples were solubilized in hot SDS-sample buffer, boiled for 3 min, subjected to 10% SDS-PAGE (Laemmli, 1970), and transferred to nitrocellulose paper (Bennett and Davis, 1981). The nitrocellulose sheets were incubated and washed as described previously (Gregorio et al., 1988). Anti-ankyrin antiserum was diluted 1:500 and the peroxidase-conjugated secondary antibody was diluted 1:1000. 4-chloro-1-napthol dissolved in methanol (3 mg ml^{-1}) and diluted 1:5 with TBS containing 0.005% H_2O_2 was used to visualize the peroxidase activity.

Indirect Immunofluorescence

Isolated lymphocytes were prepared for immunofluorescence staining as previously described (Repasky et al., 1984). When localizing both spectrin

1. *Abbreviations used in this paper*: PKC, protein kinase C; TcR, T cell receptor.

and ankyrin in the same cells, coverslips were incubated in 0.1% bovine serum albumin in PBS for 30 min at 37°C to minimize nonspecific binding of the antibodies. Cells were stained with goat anti-α-spectrin antiserum (diluted 1:50 in PBS), followed by fluorescein-conjugated sheep anti-goat IgG. The coverslips were then incubated with rabbit anti-ankyrin antiserum (1:25 in PBS) and rhodamine-conjugated goat anti-rabbit IgG. Identical results were obtained using an alternative protocol involving the following antibodies: goat anti-α-spectrin antiserum, rhodamine-conjugated donkey anti-goat IgG, rabbit anti-ankyrin antiserum and fluorescein-conjugated donkey anti-rabbit IgG. To localize both ankyrin and PKCβ in the same cells, isolated T lymphocytes were probed with mouse anti-PKCβ antibodies (7 μg ml^{-1}) followed by fluorescein-conjugated sheep anti-mouse IgG. The coverslips were then incubated in rabbit anti-ankyrin antiserum, followed by rhodamine-conjugated goat anti-rabbit IgG. For all experiments, identical results were obtained using an isotype-specific rabbit anti-PKCβII peptide antiserum which was generously provided by Dr. A. Fields (Case Western Reserve, Cleveland, OH; Hocevar and Fields, 1991).

In some experiments, cells were treated with a cytoskeletal extraction buffer (CSK) containing 1% Triton X-100 for 5 min at 4°C (Greenberg and Edelman, 1983). The extracted cells were then washed twice with CSK buffer without Triton X-100, twice with PBS, and then fixed for 20 min in PBS/4% formaldehyde. CSK-extracted cells were immunostained as described above.

The immunostained coverslips were analyzed using an Olympus BM-2 microscope; micrographs were taken using Tri-X or T-Max (400 ASA) film (Eastman Kodak Co., Rochester, NY). The percentage of cells with cytoskeletal aggregates was quantified by examining a minimum of 200 cells per group. Each value represents the mean ± SEM of a minimum of three experiments.

Ultrastructural Immunocytochemical Localization of Ankyrin and Spectrin

Lymph nodes isolated from Balb/c mice were fixed in periodate-lysine-paraformaldehyde fixative (PLP) (McLean and Nakane, 1974) for 3.5 h and cryoprotected overnight in 0.1 M phosphate buffer, pH 7.4, containing 60% sucrose. The tissue was quick frozen in liquid nitrogen; 25–30-μm sections were cut on a cryostat (AO HistoSTAT, Reichart Scientific Instruments, Buffalo, NY) at −20°C and subsequently thawed in 60% sucrose/PBS. The free-floating sections were incubated overnight in anti-ankyrin antiserum (1:15, diluted in PBS/0.1% BSA/0.005% saponin). Washes, incubations in secondary antibody, visualization of antibody binding sites, and processing for electron microscopy were carried out as described (Black et al., 1988). Control samples treated with preimmune serum showed no specific staining.

The protocol for the ultrastructural localization of spectrin in cultured lymphocytes has been described in detail previously (Black et al., 1988). For localization of ankyrin in DO-11.10 cells, cultured lymphocytes were fixed with PLP and immunostained for ankyrin as described for lymphoid tissue sections (see above), except that the fixed cells were extracted with 0.01% saponin/PBS and incubated in primary antiserum for 2 h. Before fixation in 2% glutaraldehyde, the cells were incubated in suspension; after this step all incubations were performed on pelleted cells.

Activation of Isolated Murine Lymphocytes

Isolated T lymphocytes were washed in fresh medium and stimulated at a final concentration of 2 × 10^6 ml^{-1}. The protein kinase C activator, phorbol 12-myristate 13-acetate (PMA) (Sigma Chemical Co., St. Louis, MO) was added directly to the cell suspension to obtain a final concentration of 10 ng ml^{-1}. Solvent was used as a control in each experiment. T cells were activated using an immobilized (H57-597) pan-reactive anti-mouse α,β T cell receptor (TcR) monoclonal antibody (Kubo et al., 1989). Briefly, 24-well culture plates were coated with protein A (Sigma Chemical Co.) (100 μg ml^{-1} in PBS) overnight, at 4°C. The wells were washed and 250 μl of undiluted H57-597 culture supernatant was added for 2 h at 4°C, followed by additional washes. Cells added to uncoated plates and to plates coated with protein A alone were used as controls. T cells were also activated using methods which bypass the TcR; i.e., 10 μg ml^{-1} concanavalin A (Con A) plus 50 ng ml^{-1} PMA.

Lymphocyte activation was confirmed using a standard proliferation assay; 70 h after treatment, 0.5 μC [^{3}H]TdR (Dupont-NEN, Boston, MA) was added to 2 × 10^5 cells in 300 μl of medium and the cells were harvested 6 h later. Incorporated radioactivity was determined by scintillation counting. Data are expressed as mean incorporation of [^{3}H]TdR into T cells. Values in cpms are representative of four experiments done in triplicate. Each value represents the mean ± SEM.

Protein Kinase C Inhibitors

Lymph node T cells were plated at a density of 2 × 10^6 cells ml^{-1} and treated with 10 μM staurosporine for 30 min (ICN Biomedicals, Inc.), 100 μM H-7 for 30 min (Seikagaku America Inc., Rockville, MD), or 0.5 μM calphostin C for 1 h (Kamiya Biomedical Co., Thousand Oaks, CA) before processing for immunofluorescence microscopy. A wide range of inhibitor concentrations were tested (50–400 μM H-7, 1–100 μM staurosporine and 0.05–5 μM calphostin C). Inhibitors were titrated based on the degree of cytoskeletal protein translocation and viability. All final concentrations of inhibitors used were within the range of reported doses required for inhibition of PKC. Solvent was used as a control in each experiment.

Results

Immunoblot Analysis of Lymphocyte Ankyrin

In this study, an antibody generated in rabbits against chicken erythrocyte ankyrin (goblin; isolated from two-dimensional gels) was used to characterize T lymphocyte ankyrin with regard to its subcellular distribution and response to signals involved in lymphocyte activation. Use of this antibody in Western blot analysis revealed a 260-kD band in a chicken erythrocyte preparation, a 215-kD band in a mouse erythrocyte preparation, and a 215-kD band in a sample of purified human erythrocyte ankyrin (Fig. 1, lanes *1*, *2*, and *3*, respectively). These results are consistent with reported molecular weights for ankyrin as observed on SDS-gels in these tissues (Bennett and Stenbuck, 1979; Yu and Goodman, 1979; Luna et al., 1979; Alper et al., 1980). The same antiserum detected a 72-kD band as well as a 215-kD band in a sample enriched for T lymphocytes (Fig. 1, lane *4*). The relative proportions of the lymphocyte 215- and 72-kD bands varied significantly from experiment to experiment. In all experiments the 72-kD band was predominant (see also: Bourguignon et al., 1986). However, when conditions were optimized to reduce proteolysis and electrophoretic-transfer was carried out under conditions which facili-

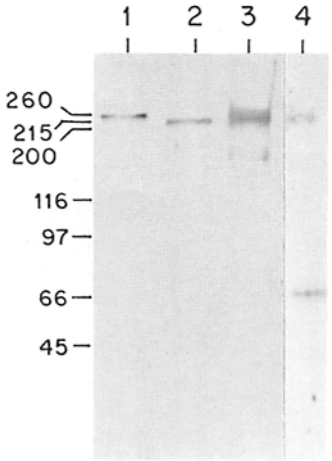


Figure 1. Western blot analysis of murine T lymphocytes using rabbit anti-chicken erythrocyte ankyrin antiserum reveals cross-reactive bands of 215 and 72 kD. Samples included chicken erythrocyte membranes (lane *1*), murine erythrocyte membranes (lane *2*), a preparation of purified human erythrocyte ankyrin (lane *3*), and an enriched population of Balb/c lymph node T lymphocytes (lane *4*). The antiserum detected a 260-kD band in chicken erythrocyte membranes (goblin), a 215-kD cross-reactive band in murine and human erythrocytes, and cross-reactive bands of 215 and 72-kD in murine T lymphocytes. The numbers in the left margin represent the relative molecular mass standards (kD).

tate transfer of high molecular weight proteins, more of the 215-kD band could be detected (Fig. 1, lane *4*).

Ankyrin Distribution in Isolated T Lymphocytes: Comparison with the Distribution of Lymphocyte Spectrin and PKCβ

To examine the subcellular distribution of ankyrin in lymphocytes, T cells isolated from murine lymph nodes were stained by indirect immunofluorescence using the polyclonal anti-ankyrin antiserum described above. Extensive heterogeneity was observed in the localization of ankyrin in these cells (Fig. 2, *a* and *c*). While some cells showed ankyrin uniformly distributed at the plasma membrane or restricted to a distinct membrane-associated "cap," others showed the protein accumulated in small patches or in a single large aggregate in the cytoplasm. In addition, depending on the plane of focus, a filamentous star-like staining pattern could be seen in the cytoplasm of every cell (e.g., Fig. 2 *c*, *open arrow*; for a particularly clear example of this staining pattern see Fig. 7 *C,i*). The specificity of the ankyrin staining patterns, including the filamentous pattern described above, was confirmed in competition experiments using purified erythrocyte ankyrin. A 50-fold molar excess of purified ankyrin included in the primary antibody incubation step eliminated all staining (data not shown).

To determine whether ankyrin colocalizes with spectrin and PKCβ in T lymphocytes, double immunofluorescence studies were performed. Fig. 2 demonstrates that ankyrin colocalizes precisely with each of these proteins, except in the case of the star-like staining pattern which appears to be unique to ankyrin (see Discussion).

The heterogeneity in the subcellular distribution of lymphocyte ankyrin and its ability to accumulate in an intracellular aggregate were unexpected based on its tight association with the plasma membrane in other cell types. To characterize further the nature of lymphocyte ankyrin, and to gain insight into the basis for the heterogeneity in its distribution, in vivo and in vitro model systems were used for (*a*) detailed examination of its organization at the ultrastructural level of resolution and (*b*) analysis of its organization in relation to lymphocyte function.

Ultrastructural Localization of Lymphocyte Ankyrin

Ultrastructural immunoperoxidase localization of ankyrin in sections of murine lymph node revealed that cytoplasmic aggregates of ankyrin can occur several microns from the cell

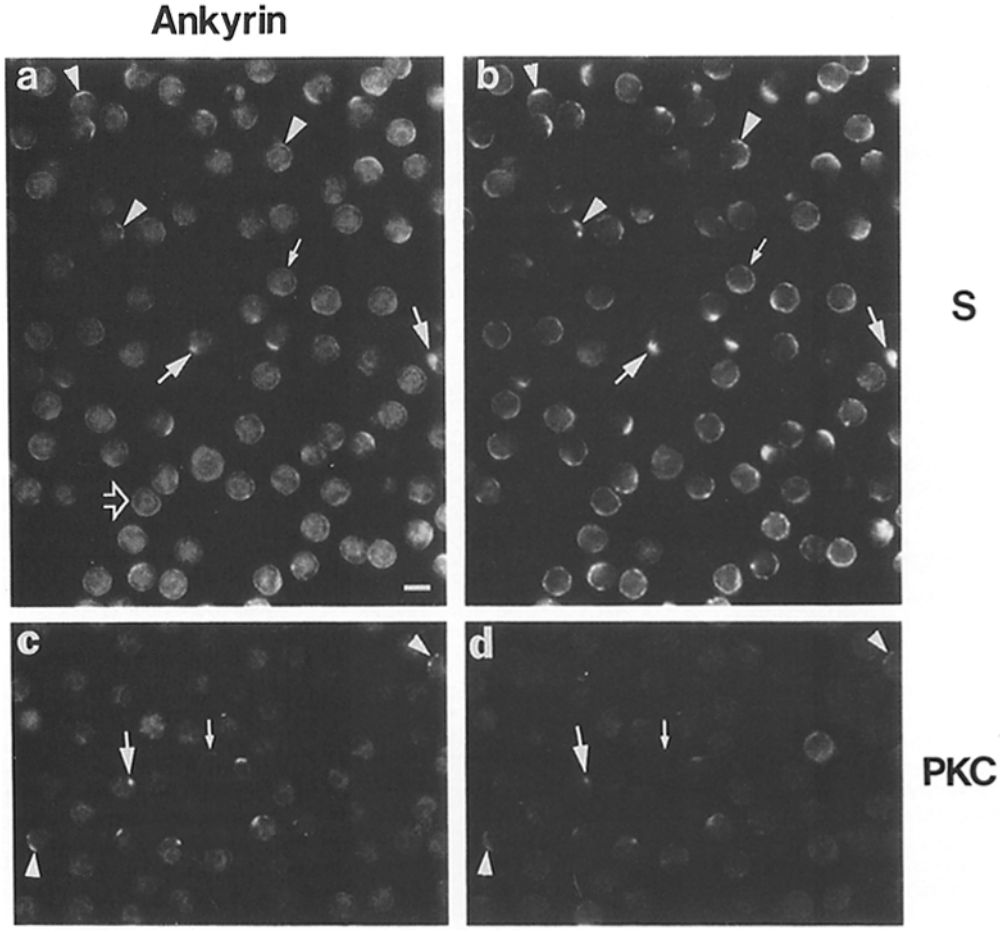


Figure 2. Subcellular distribution of ankyrin in isolated T lymphocytes: comparison with the distribution of lymphocyte spectrin and PKCβ. Untreated freshly isolated murine T lymphocytes were stained for ankyrin using rabbit anti-chicken erythrocyte ankyrin (goblin) antiserum (*a* and *c*) as described in Materials and Methods. Double immunofluorescence techniques were used to compare the localization of ankyrin and spectrin (*S*) (*a* and *b*, respectively) and of ankyrin and PKCβ (*c* and *d*, respectively) in the same cells. The subcellular distribution of ankyrin varied considerably among tissue T cells: while some cells showed the expected "ring" of immunofluorescence staining at the plasma membrane (*small arrow*), others showed a membrane-associated polar cap or several small patches at the membrane and/or in the cytoplasm (*arrowheads*), and many showed all of the protein accumulated in a single large cytoplasmic aggregate (*large arrows*). In addition, all cells exhibited a filamentous star-like staining pattern for ankyrin (*open arrow*). Ankyrin was found to colocalize precisely with spectrin and PKCβ in T cells, except in the case of the filamentous staining pattern which was displayed only by ankyrin. Note, that *c* and *d* (*middle, right side*) contain a contaminating cell which does not demonstrate colocalization of PKCβ with ankyrin. Bar, 10 μM.

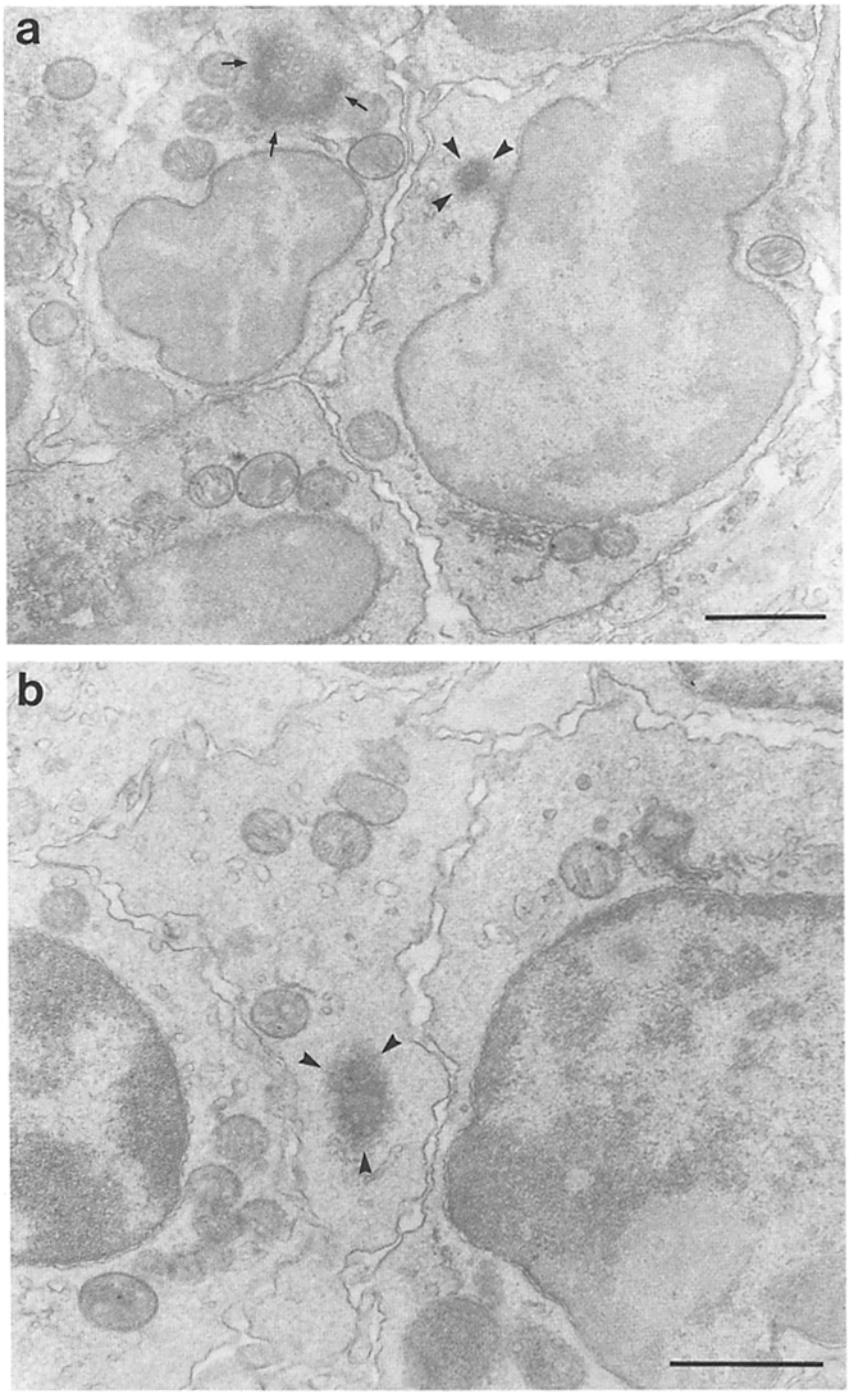


Figure 3. Ultrastructural immunoperoxidase localization of ankyrin in murine lymphocytes in situ. PLP-fixed sections of murine lymph node were immunostained for ankyrin at the ultrastructural level using a pre-embedding immunoperoxidase technique. Immunoprecipitate can be seen in the cytoplasm of several lymphocytes, at some distance from the plasma membrane (*arrowheads*). Many lymphocytes contain a discrete aggregate of ankyrin near the nucleus or Golgi apparatus. Note the numerous membranous vesicles present within the region demonstrating ankyrin immunoprecipitate. The aggregate is generally oval or spherical in shape, although it can be horseshoe-shaped (*arrows*). Bar, 1 μM.

surface (Fig. 3, *a* and *b*). The aggregates were generally spherical or oval in shape although, as previously observed for lymphocyte spectrin aggregates (Black et al., 1988), they could also be horseshoe-shaped (Fig. 3 *a*). Numerous membranous vesicles were present within the area demonstrating ankyrin immunoprecipitate. No staining was observed at the plasma membrane; this is likely to be due to sensitivity limitations of the pre-embedding immunostaining technique and the overall low levels of ankyrin in lymphocytes (data not shown) since, as shown in Fig. 2, many lymphocytes do express ankyrin at the cell periphery.

The aggregate of ankyrin was further characterized in the DO.11-10 cell line, a major histocompatibility complex-restricted, functionally mature T lymphocyte hybridoma specific for ovalbumin (Kappler et al., 1981; Haskins et al., 1983). These cells constitutively and homogeneously express a large cytoplasmic aggregate of spectrin (Black et al., 1988; Lee et al., 1988). The basis for the constitutive expression of the aggregate phenotype (one of the distribution patterns of spectrin known to occur in situ) in these long-term cultured cells, which exhibit transformed or leukemic properties, is unknown. However, they were useful for immunolocalization studies at the light and electron microscope level of resolution to assess the relationship between the ankyrin and spectrin aggregates.

Using immunofluorescence localization techniques, anky-

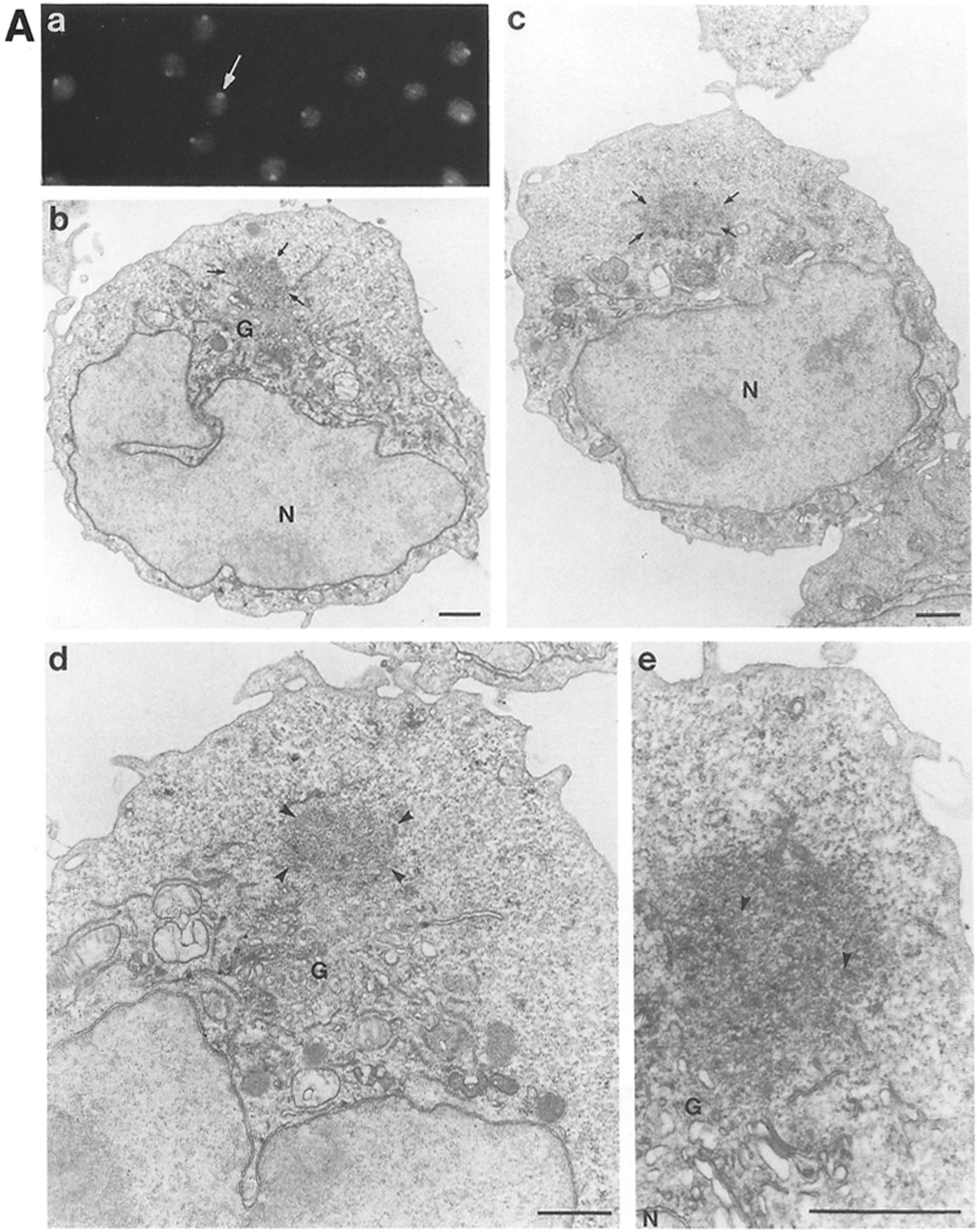


Figure 4.

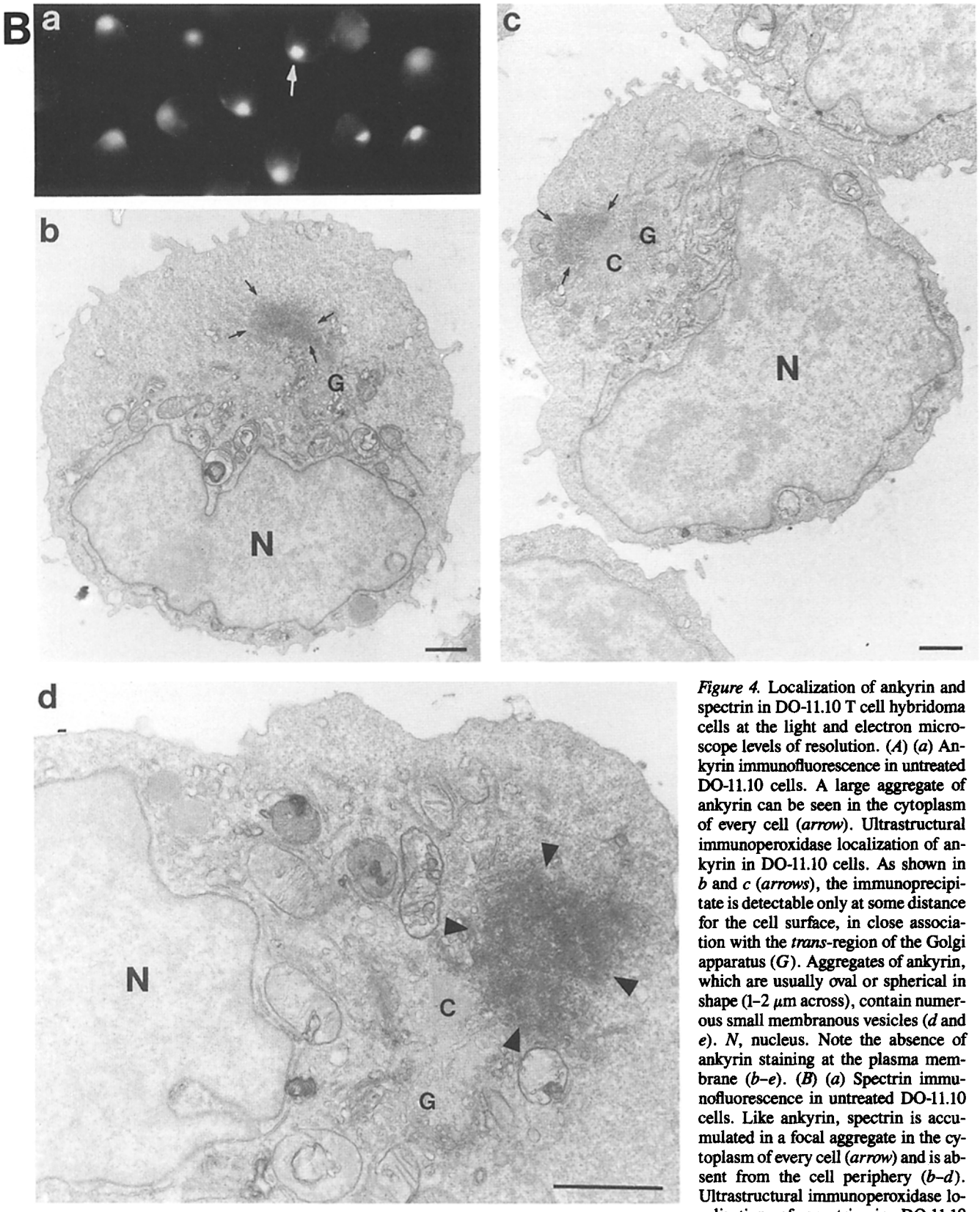


Figure 4. Localization of ankyrin and spectrin in DO-11.10 T cell hybridoma cells at the light and electron microscope levels of resolution. (*A*) (*a*) Ankyrin immunofluorescence in untreated DO-11.10 cells. A large aggregate of ankyrin can be seen in the cytoplasm of every cell (*arrow*). Ultrastructural immunoperoxidase localization of ankyrin in DO-11.10 cells. As shown in *b* and *c* (*arrows*), the immunoprecipitate is detectable only at some distance for the cell surface, in close association with the *trans*-region of the Golgi apparatus (*G*). Aggregates of ankyrin, which are usually oval or spherical in shape (1–2 μm across), contain numerous small membranous vesicles (*d* and *e*). *N*, nucleus. Note the absence of ankyrin staining at the plasma membrane (*b–e*). (*B*) (*a*) Spectrin immunofluorescence in untreated DO-11.10 cells. Like ankyrin, spectrin is accumulated in a focal aggregate in the cytoplasm of every cell (*arrow*) and is absent from the cell periphery (*b–d*). Ultrastructural immunoperoxidase localization of spectrin in DO-11.10 cells. Reaction product (*arrows*) can be seen accumulated near the *trans*-region of the Golgi apparatus and centriolar complex (*C*). The spectrin aggregate is also oval or spherical in shape, measures 1–2 μm across, and is packed with small membranous vesicles. These micrographs show that the ankyrin and spectrin aggregates in lymphocytes are indistinguishable with regard to shape, size and subcellular localization. Bars: (*A,a* and *B,a*) 10 μm; (*A,b–d* and *B,b–d*) 1 μm.

rin was also found to be expressed as a cytoplasmic aggregate in DO-11.10 cells (Fig. 4 *A,a*; compare with Fig. 4 *B,a*). Double immunofluorescence studies revealed that spectrin and ankyrin are co-localized in these cells (data not shown). Ultrastructural immunoperoxidase staining revealed that, like spectrin (Fig. 4 *B,b–d*), ankyrin is also accumulated in a discrete region at one pole of the DO.11-10 cell, several microns from the cell surface and usually in close association with the centriolar complex and the *trans*-region of the Golgi apparatus (Fig. 4 *A,b–e*). Numerous membranous vesicles of various sizes could be seen clustered within the ankyrin aggregate (Fig. 4 *A,e, arrowheads*); note that these vesicles were also present within the area stained for spectrin (Fig. 4 *B,d*). The location of the spectrin and ankyrin reaction product corresponds well to the location of the cytoplasmic structure composed primarily of a fine meshwork of densely packed filaments, as observed by conventional thin-section microscopy (i.e., in the absence of immunoperoxidase staining, data not shown; see Black et al., 1988). The cytoskeletal aggregate was not membrane bound; however, it excluded most other cellular organelles. Since immunoperoxidase localization of either spectrin or ankyrin resulted in identical staining patterns, we conclude that the previously characterized structure in DO-11.10 cells that contains spectrin (Black et al., 1988) also contains ankyrin.

Ankyrin Is a Dynamic Molecule in Lymphocytes: Effects of Activation-related Signals on T Lymphocyte Ankyrin Distribution

The finding that ankyrin, spectrin and PKCβ are colocalized in lymphocytes (see above), together with the observation that the distribution of lymphocyte spectrin can be altered by activation-related signals (Lee et al., 1988; Gregorio et al., 1992), led us to examine the effects of direct PKC stimulation and lymphocyte activation on the organization of ankyrin in tissue-derived T lymphocytes. The phorbol ester PMA was used to stimulate PKC directly. Treatment of T lymphocytes with this agent resulted in a loss of the naturally occurring heterogeneity in lymphocyte ankyrin distribution and a dramatic reorganization of this protein into a single large cytoplasmic aggregate (compare untreated cells in Fig. 5, *a* and *g* with PMA-treated cells in Fig. 5, *c* and *i*). Although not apparent in the micrograph shown, it should be noted that the filamentous star-like distribution of ankyrin staining was unaffected by PMA treatment; the significance of this localization was, therefore, not addressed in this study (see Discussion). Within 5 min of the addition of PMA, the fraction of cells containing an aggregate of ankyrin increased from ~20 to 50%, and reached >75% within 30 min. This striking alteration in the distribution of ankyrin was accompanied by a coincident reorganization of lymphocyte spectrin (Fig. 5, *a–d*) and PKCβ (Fig. 5, *g–j*).

To determine whether the cytoplasmic aggregates of ankyrin, spectrin and PKCβ which appeared after PKC stimulation were formed from newly synthesized protein, tissue-derived T cells were pretreated with 10 μg ml^{-1} cycloheximide prior to the addition of PMA. Appearance of aggregates was not sensitive to this inhibitor of protein synthesis (Table I), indicating that they are formed by the translocation of preexisting protein. This experiment strongly suggests that the movement of ankyrin, spectrin and PKCβ within the lymphocyte cytoplasm is posttranslationally regulated.

Since PKC has been shown to be an important second messenger triggered by activation of the T cell antigen receptor, we speculated that lymphocyte ankyrin may be functionally linked to activation-induced events. To investigate this hypothesis, T lymphocytes were activated via the T cell receptor (TcR) complex using an immobilized pan-reactive anti-mouse $\alpha\beta$ T cell receptor-specific monoclonal antibody (H57-597; Kubo et al., 1989) or by methods which bypass the TcR such as a combination of PMA and Con A. Activation using either of these methods also resulted in a rapid and long-lasting increase in the percentage of T cells containing large, coincident aggregates of ankyrin, spectrin and PKCβ (Fig. 5, *e* and *f* [TcR simulation]; Fig. 5, *k* and *l* [PMA plus Con A stimulation]).

To examine the solubility properties of aggregated ankyrin, lymph node-derived T lymphocytes were treated with a Triton X-100–containing cytoskeleton-stabilizing buffer (CSK buffer) reported to extract the cytoplasm and leave behind the cytoskeleton, nucleus and some cytoskeleton-associated proteins (Greenberg and Edelman, 1983). When freshly isolated T lymphocytes are treated with CSK, the majority of cells exhibited little or no staining for ankyrin, spectrin or PKCβ (indicating for example, that membrane-associated ring-like staining is Triton X-100 soluble); however, cytoplasmic aggregates and membrane caps of these proteins were evident in some cells. In contrast, virtually all T lymphocytes extracted with CSK after PMA treatment exhibited large aggregates of detergent-insoluble ankyrin, spectrin and PKCβ (Fig. 6). The percentage of control or PMA-treated T cells containing these proteins in a cytoplasmic aggregate was unaffected by CSK extraction, suggesting that all aggregates are Triton X-100 insoluble.

Effects of Protein Kinase C Inhibitors on Ankyrin Organization in T Lymphocytes

The data described above suggest that the organization of lymphocyte ankyrin and spectrin is related to that of lymphocyte PKCβ and suggest that the presence of coincident cytoplasmic aggregates of these proteins in situ may be indicative of physiological stimulation of PKC. To gain further insight regarding the relationship between these proteins in lymphocytes, the effects of several inhibitors of PKC on their organization in murine T cells were examined. Three PKC inhibitors were used for these studies including staurosporine, H-7 and calphostin C. Staurosporine and H-7 have been reported to act on the catalytic domain of PKC (Nakadate et al., 1988; Hidaka et al., 1984), whereas the highly specific PKC inhibitor, calphostin C interacts with the regulatory domain of the enzyme and inhibits the binding of diacylglycerol analogues (Kobayashi et al., 1989).

Treatment of freshly isolated tissue-derived T lymphocytes with any of the three inhibitors resulted in an alteration of the naturally occurring heterogeneity in the distribution of ankyrin, spectrin and PKCβ. Treatment with staurosporine, H-7 or calphostin C resulted in a steady increase in the fraction of cells exhibiting ankyrin, spectrin and PKCβ at the cell periphery and a decrease in the fraction of cells expressing cytoplasmic aggregates or patches of these proteins

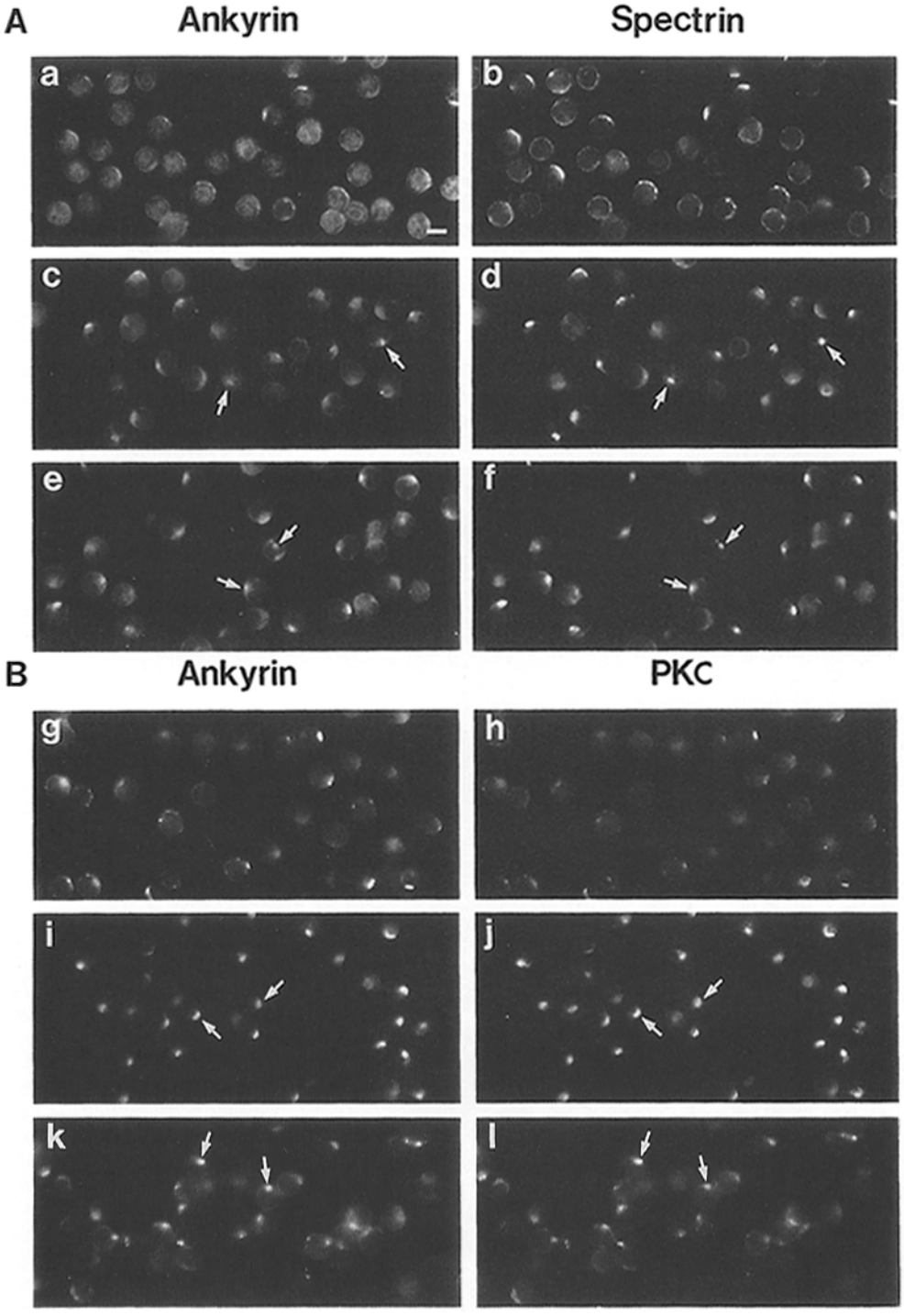


Figure 5. Phorbol ester treatment or activation of lymph node–derived T lymphocytes results in a rapid accumulation of ankyrin, spectrin and PKCβ in an intracellular aggregate. T lymphocytes were isolated from murine lymph nodes by passage of cell suspensions over nylon wool. Control and treated cells were double-stained for ankyrin and spectrin (*A*) or for ankyrin and PKCβ (*B*) using indirect immunofluorescence techniques. Ankyrin immunofluorescence is always shown on the left. Micrographs show distribution patterns after treatment of cells for 20 min. (*A*) (*a* and *b*) untreated T lymphocytes; (*c* and *d*) T cells treated with PMA (10 ng ml^{-1}) to stimulate PKC directly; (*e* and *f*) T cells activated through the antigen receptor using an immobilized pan-reactive anti-mouse $\alpha\beta$ TcR-specific monoclonal antibody (H57-597). (*B*) (*g* and *h*) untreated T lymphocytes; (*i* and *j*) T cells treated with PMA (10 ng ml^{-1}); (*k* and *l*) T cells activated using a combination of PMA and Con A (50 ng ml^{-1} and 10 μg ml^{-1}, respectively). Stimulation of lymphocyte PKC with phorbol ester or activation of T cells using two different methods resulted in rapid disappearance of the naturally occurring heterogeneity in the distribution of ankyrin, spectrin and PKCβ in lymphocytes and accumulation of these proteins in a large cytoplasmic aggregate. Arrows demonstrate colocalization of proteins in such aggregates. T cell activation was confirmed by measuring [^{3}H]thymidine uptake: control no. 1 (no additions), 653 ± 358 cpm; control no. 2 (protein A alone), 1,447 ± 210 cpm; H57-597 activated, 77,076 ± 9,697 cpm; and conA plus PMA activated, 333,311 ± 10,790 cpm. Bar, 10 μM.

(compare Fig. 7, *A–C* with Fig. 2 [untreated control cells]). The pattern of membrane-associated immunofluorescence staining differed, however, with the drug used. In T lymphocytes treated with staurosporine (Fig. 7 *A*), all three proteins appeared to be accumulated in a crescent-shaped region near the plasma membrane (clearly distinct from the cytoplasmic aggregates of these proteins described above; see Figs. 5 and 6), whereas cells treated with H-7 generally exhibited uniform or interrupted rings of membrane-associated fluorescence staining (Fig. 7 *B*). Treatment of cells with calphostin

Table I. Effect of Cycloheximide (CH) on the Translocation of Lymphocyte Ankyrin, Spectrin, and PKCβ in Murine T Lymphocytes

Treatment	Percent of cells with aggregates
None	24 ± 15.0
CH	25 ± 6.4
PMA	85 ± 5.3
CH + PMA	91 ± 6.1

2×10^6 T cells ml^{-1} were preincubated for 2 h in 15 μg ml^{-1} of cycloheximide (a concentration known to inhibit protein synthesis and to abrogate the capacity of IFN-α to increase the focal distribution of spectrin in human leukemic B cells: Evans et al., 1993), before the addition of PMA. Cells were harvested 1 h after stimulation and processed for immunofluorescence microscopy. Cell viability was assessed by trypan blue exclusion. The percentage of cells with cytoskeletal aggregates was quantified by examining a minimum of 200 cells per group. Each value represents the mean ± SEM of three experiments.

C resulted in an increase in the percentage of cells with plasma membrane-associated rings of fluorescence staining (Fig. 7 *C*). The fact that ankyrin, spectrin and PKCβ underwent an identical reorganization in response to treatment with these inhibitors provides additional support for a functional association of these proteins in T lymphocytes.

Discussion

The studies described in this report demonstrate that lymphocyte ankyrin can be stably expressed at some distance from the plasma membrane and can redistribute throughout the cytoplasm in response to physiological T lymphocyte activation-related signals. The extensive heterogeneity in the subcellular distribution of ankyrin both in lymphoid tissues and in lymphocyte cell lines reflects the dynamic nature of ankyrin. While ankyrin is often found at the lymphocyte plasma membrane, as would be expected for a protein that can link cell surface macromolecules to the underlying membrane cytoskeleton, it can also be found accumulated in a single large cytoplasmic aggregate or in numerous smaller patches in the cytoplasm or near the nucleus. Ultrastructural localization studies show that the cytoplasmic aggregate of lymphocyte ankyrin is a discrete, nonmembrane-bound structure containing numerous minute membranous vesicles. It is

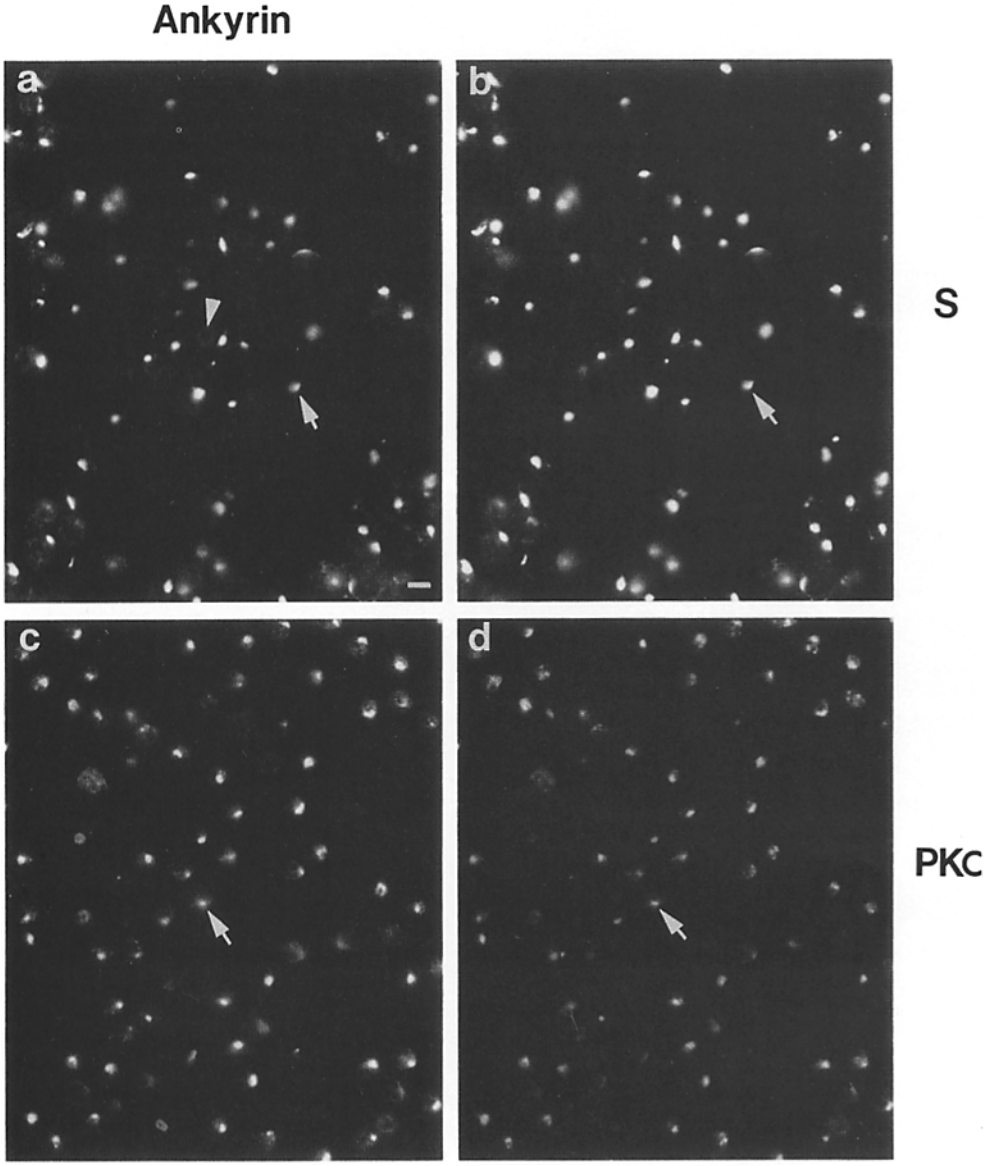


Figure 6. Colocalization of ankyrin, spectrin, and PCKβ in a Triton X-100–insoluble aggregate induced in T lymphocytes by PMA treatment. Isolated T cells were treated with PMA (10 ng ml^{-1}) to induce the formation of cytoskeletal aggregates. Treated cells were immobilized on Alcian blue–coated coverslips, extracted with a cytoskeleton stabilization buffer containing 1% Triton X-100, fixed in 4% formaldehyde and double-stained for ankyrin and spectrin (*S*) (*a* and *b*, respectively) or ankyrin and PKCβ (*c* and *d*, respectively) using indirect immunofluorescence techniques. Arrows indicate detergent insoluble aggregates of ankyrin, spectrin and PKCβ. Bar, 10 μM.

generally found several microns from the plasma membrane, and can frequently be seen in close association with the Golgi apparatus. Since, in DO-11.10 cells, the cytoplasmic region stained by anti-ankyrin antibodies is indistinguishable with regard to localization, shape, size and ultrastructural appearance from that observed using anti-spectrin antibodies, we conclude that ankyrin is a component of the previously characterized "spectrin-rich structure" (Black et al., 1988).

Since double immunofluorescence studies presented here clearly show that aggregated and membrane-associated lymphocyte ankyrin and spectrin are always colocalized with PKCβ, we propose that PKCβ is also a component of the cytoskeletal aggregate (also see Gregorio et al., 1992). This observation is of particular importance since PKC is an integral component of the phosphatidylinositol cascade, which is involved in a wide range of cellular functions including T cell activation (Nishizuka, 1988). Stimulation of the antigen receptor leads to phosphatidylinositol hydrolysis and liberation of two second messengers, one of which (diacylglycerol) is a potent activator of PKC (Berridge and Irvine, 1989). The colocalization of PKCβ with cytoskeletal elements in the lymphocyte cytoplasm suggests the interesting possibility that structural proteins play a role in signal transduction mechanisms in this cell type.

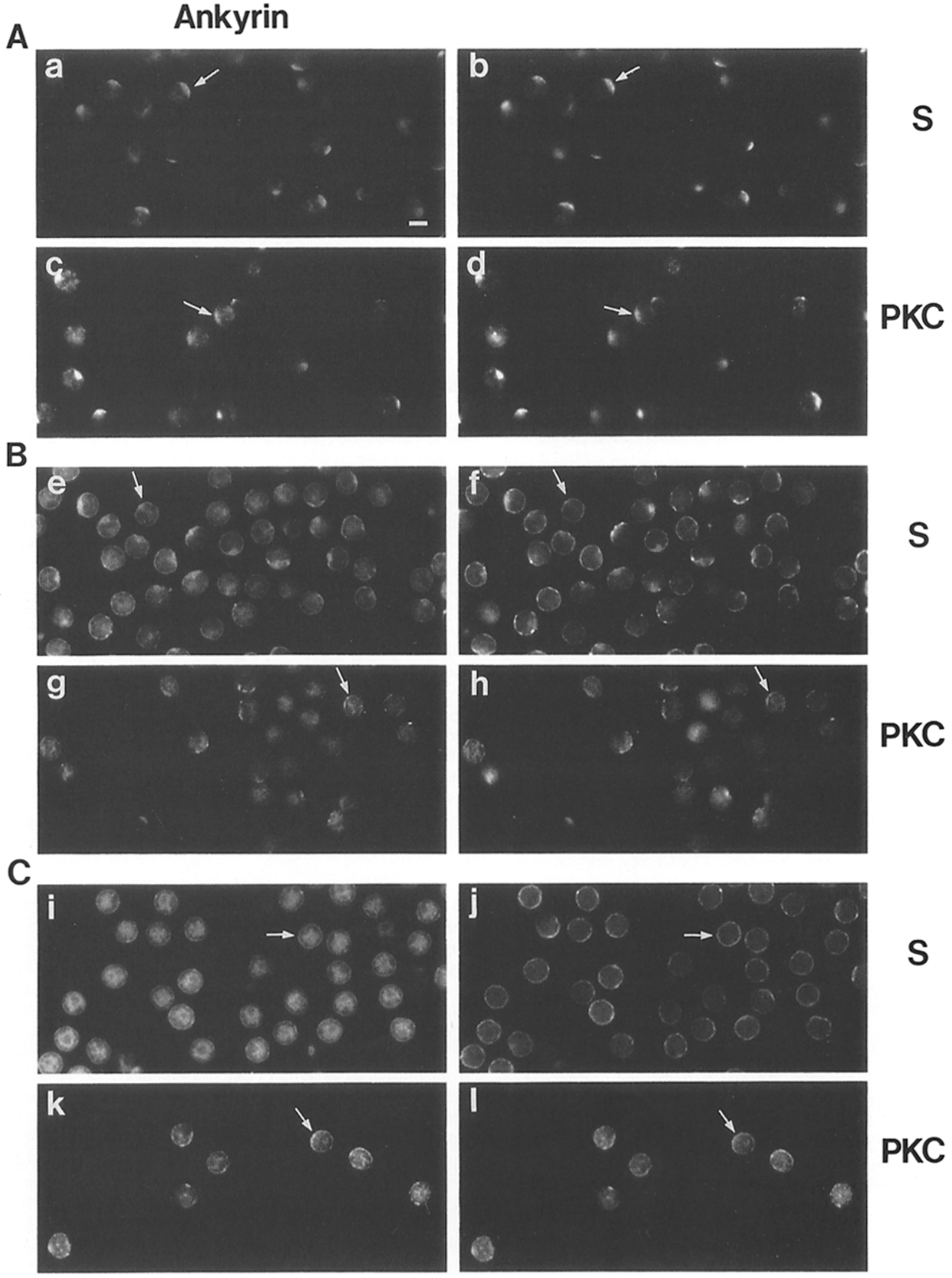
Insight regarding the basis for the heterogeneity in lymphocyte ankyrin distribution was obtained from examination of the effects of activation-related signals on its subcellular organization in T lymphocytes. These studies clearly demonstrated (*a*) the dynamic nature of lymphocyte ankyrin and (*b*) the relationship between ankyrin subcellular distribution and PKC activity. Activation of PKC with the phorbol ester PMA or through activation of the T lymphocyte receptor (an event which has been shown to lead to PKC activation, see review: Cambier, 1992) resulted in a rapid reorganization of ankyrin in the lymphocyte cytoplasm. Ankyrin, together with spectrin and PKCβ, accumulated in a single cytoplasmic aggregate in virtually all treated cells. The rapidity with which the changes occurred (<5 min), together with their insensitivity to cycloheximide treatment, was indicative of redistribution of existing protein. Conversely, ankyrin, spectrin and PKCβ were found to move coincidentally to membrane-associated locations in response to inhibitors of the catalytic and regulatory domains of PKC, providing further evidence for a functional, and perhaps also structural, association of these proteins in the lymphocyte cytoplasm. Moreover, pretreatment with an inhibitor of the regulatory domain of PKC, calphostin C blocked the formation of cytoplasmic aggregates of ankyrin, spectrin and PKCβ in response to PMA treatment or TcR-mediated lymphocyte activation (data not shown for ankyrin; see Gregorio et al., 1992 for data on spectrin and PKCβ). Together, these data suggest that the activity and/or the conformation of the enzyme are important in maintaining appropriate distribution patterns for ankyrin, spectrin and PKCβ in lymph node T cells. With regard to the movement of these proteins to the cell periphery in response to treatment with staurosporine or H-7, it should be noted that these inhibitors are known to affect other protein kinases with sequence homology to PKC in their catalytic domain (Kobayashi et al., 1989); hence, the morphological changes observed as a result of treatment with these agents may not be a direct effect of their inhibition of PKC activity.

It is interesting to note that while freshly isolated T cells contain several different populations of T cells (i.e., $CD4^+$, $CD8^-$, $CD4^-$ $CD8^+$) virtually all of the cells responded similarly to treatment with activators or inhibitors of PKC activity with respect to the distribution of ankyrin, spectrin and PKCβ. In addition, the results described above suggest that the presence of a cytoplasmic aggregate of ankyrin, spectrin and PKCβ in T cells indicates the occurrence of an event involving activation of PKC (specifically of the β isoform), and thus provide a possible basis for one of the naturally occurring distribution patterns observed for ankyrin in T lymphocytes in situ.

Immunofluorescence staining of aggregated ankyrin, spectrin, and PKCβ in PMA-treated T lymphocytes was unaffected by exposure to a Triton-containing cytoskeleton stabilization buffer, suggesting that these proteins are part of a stable assembly of Triton-insoluble elements. Since activation of PKC is generally reflected in its translocation from the soluble to the operationally defined "particulate fraction" of the cell (consisting of plasma membrane, other membranous compartments and cytoskeletal elements; for review see Berry and Nishizuka, 1990), it is possible that the appearance of lymphocyte PKCβ in the "insoluble fraction" (also see Gregorio et al., 1992) after activation is the result of its direct or indirect interaction with detergent-insoluble cytoskeletal elements such as ankyrin and spectrin.

Immunofluorescence analysis of ankyrin distribution in T lymphocytes also revealed a filamentous "star-like" staining pattern which appears to be independent of spectrin and PKCβ. Although the basis for this "star-like" staining pattern is unknown, preliminary results from double immunofluorescence studies indicate it coincides with that of lymphocyte tubulin (data not shown). This finding is of interest since (*a*) tubulin has been shown to bind ankyrin repeats in the NH_2-terminal domain of the molecule (i.e., the 89–95-kD domain) and (*b*) it has been suggested that ankyrin may be involved in mediating attachment of tubulin to certain intracellular membranes (Davis and Bennett, 1984; Davis et al., 1991).

Immunoblot analysis of ankyrin demonstrated the presence of immunoreactive bands of 215 and 72 kD in murine tissue lymphocyte preparations, as previously seen in BW5147 cells (Bourguignon et al., 1986). The 72-kD band was predominant in all experiments, even in the presence of a variety of protease inhibitors (including the calpain-1 inhibitor calpeptin; data not shown). In view of the sensitivity of erythrocyte ankyrin to proteolysis (Bennett and Stenbuck, 1979), it is possible that the 72-kD band corresponds to the spectrin-binding domain of ankyrin (Bennett, 1992) and is a degradation fragment of the 215-kD band. In this regard, peripheral blood buffy coats have been reported to contain a protease to which ankyrin is particularly susceptible (Bennett, 1985). However, we cannot rule out the possibility that the 72-kD band represents a form of ankyrin that is present in situ and performs specific function(s) in lymphocytes, perhaps reflecting the dynamic properties of ankyrin in this cell type. The levels of the 215-kD band varied significantly from experiment to experiment, as did the relative proportions of the two immunoreactive forms of ankyrin detected.


Ankyrin
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Similar variability in the relative levels of these forms was observed after treatment with PKC activators or inhibitors, making it impossible to determine if their presence is related to the activation status of the enzyme. Further studies are required to determine whether the 72-kD immunoreactive band results from alternatively spliced messenger RNA (Hall and Bennett, 1987; Lux et al., 1990), specific proteolytic processing (Davis et al., 1989; Davis et al., 1992) or is an artifact of sample preparation.

In keeping with its function in molecular recognition (Bennett, 1992), lymphocyte ankyrin may participate in the formation of protein assemblies required to perform specific functions at restricted subcellular sites. As mentioned above, signal transducing molecules like PKCβ may come into contact with their substrates by associating directly or indirectly with cytoskeletal proteins such as ankyrin and spectrin. In accordance with its structural role in erythrocytes and with its reported function in subcellular targeting events, ankyrin (perhaps in association with spectrin) is ideally suited to provide the scaffolding necessary for the appropriate placement of PKCβ for phosphorylation of molecules involved in T cell activation or other lymphocyte functions. It is noteworthy that ankyrin has been shown to be a phosphoprotein (Alper et al., 1980). To date, however, neither ankyrin nor spectrin has been identified as a substrate for PKC in any system, although nonerythroid β_G- and β_R-spectrins have recently been reported to contain domains with amino acid sequence homology to pleckstrin, a major protein kinase C substrate (Haslam et al., 1993).

The ability of lymphocyte ankyrin to be stably expressed as a detergent-insoluble aggregate at some distance from the plasma membrane and to relocate to discrete cytoplasmic sites may represent properties that are unique to the lymphocyte form of the molecule. Interaction of lymphocyte ankyrin with proteins that are not always localized at the cell surface adds an additional complexity to this molecule. Our data on tissue derived lymphocytes suggest that previous work on the association of ankyrin with a glycoprotein (gp85) at the plasma membrane in one T lymphoma cell line (Kalomiris and Bourguignon, 1988; Lokeshwar and Bourguignon, 1991; Bourguignon et al., 1991) may underrepresent the significance of lymphocyte ankyrin's role in T cell function. Its relocation to a site at some distance from the lymphocyte plasma membrane in response to important physiological (i.e., activation related) signals indicates that ankyrin is not solely involved in anchorage of proteins at the plasma membrane in lymphoid cells.

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Figure 7. Treatment of murine T lymphocytes with the PKC inhibitors staurosporine, H-7 or calphostin C results in a co-translocation of ankyrin, spectrin and PKCβ to the cell periphery, with a concomitant loss of the natural heterogeneity in the distribution of the proteins in these cells. T lymphocytes were treated with (*A*) staurosporine (10 μM) or (*B*) H-7 (100 μM) for 30 min, or (*C*) calphostin C (0.5 μM) for 2 h. After treatment, cells were fixed in 2% formaldehyde and double-immunostained for ankyrin and spectrin (*S*) (*a* and *b*, *e* and *f*, *i* and *j*) or ankyrin and PKCβ (*PKC*) (*c* and *d*, *g* and *h*, *k* and *l*). Ankyrin immunofluorescence is always shown on the left. Treatment with staurosporine (*A*) resulted in the accumulation of ankyrin, spectrin and PKCβ in a crescent-shaped region near the plasma membrane (*arrows*). T cells treated with H-7 (*B*) generally exhibited uniform or interrupted rings of membrane-associated fluorescence staining for these proteins (*arrows*). Calphostin C-treated cells (*C*) also displayed rings of fluorescence staining (*arrows*). Approximately 2 h after treatment with any of the inhibitors, <90% of treated cells exhibited the inhibitor-induced staining pattern. Bar, 10 μM.

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