Spectrin Redistributes to the Cytosol and Is Phosphorylated during Mitosis in Cultured Cells

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Abstract. Dramatic changes in morphology and extensive reorganization of membrane-associated actin filaments take place during mitosis in cultured cells, including rounding up; appearance of numerous actin filament–containing microvilli and filopodia on the cell surface; and disassembly of intercellular and cell–substratum adhesions. We have examined the distribution and solubility of the membrane-associated actin-binding protein, spectrin, during interphase and mitosis in cultured CHO and HeLa cells. Immunofluorescence staining of substrate-attached, well-spread interphase CHO cells reveals that spectrin is predominantly associated with both the dorsal and ventral plasma membranes and is also concentrated at the lateral margins of cells at regions of cell–cell contacts. In mitotic cells, staining for spectrin is predominantly in the cytoplasm with only faint staining at the plasma membrane on the cell body, and no discernible staining on the membranes of the microvilli and filopodia (retraction fibers) which protrude from the cell body. Biochemical analysis of spectrin solubility in Triton X-100 extracts indicates that only 10–15% of the spectrin is soluble in interphase CHO or HeLa cells growing attached to tissue culture plastic. In contrast, 60% of the spectrin is soluble in mitotic CHO and HeLa cells isolated by mechanical "shake-off" from nocodazole-arrested synchronized cultures, which represents a four- to sixfold increase in the proportion of soluble spectrin. This increase in soluble spectrin may be partly due to cell rounding and detachment during mitosis, since the amount of soluble spectrin in CHO or HeLa interphase cells detached from the culture dish by trypsin-EDTA or by growth in spinner culture is 30–38%. Furthermore, mitotic cells isolated from synchronized spinner cultures of HeLa S3 cells have only 2.5 times as much soluble spectrin (60%) as do synchronous interphase cells from these spinner cultures (25%).

The β subunit of spectrin is phosphorylated exclusively on serine residues both in interphase and mitosis. Comparison of steady-state phosphorylation levels of spectrin in mitotic and interphase cells demonstrates that solubilization of spectrin in mitosis is correlated with a modest increase in the level of phosphorylation of the spectrin β subunit in CHO and HeLa cells (a 40% and 70% increase, respectively). Two-dimensional phosphopeptide mapping of CHO cell spectrin indicates that this is due to mitosis-specific phosphorylation of β-spectrin at several new sites. This is independent of cell rounding and dissociation from other cells and the substratum, since no changes in spectrin phosphorylation take place when cells are detached from culture dishes with trypsin-EDTA. We propose that mitosis-specific redistribution of spectrin to the cytosol is a consequence of phosphorylation by mitotic kinases and reflects spectrin dissociation from the plasma membrane. This could be functionally related to restructuring of membrane-associated actin filament networks in mitotic cells and could play a role in recruitment of plasma membrane to microvilli and filopodia as well as in reduction of cell–cell or cell–substratum contacts in mitotic cells.

D RAMATIC changes in morphology and cell surface properties take place during mitosis in animal cells in culture, including rounding up, appearance of microvilli, increase in cortical stiffness, and reduction in cell–cell and cell–substratum contacts (Porter et al., 1973; Salmon, 1989). In addition, mitotic cells are incapable of regulated or constitutive exocytosis, phagocytosis, and receptor-mediated or fluid-phase endocytosis (Warren, 1985). Extensive reorganization of cortical and membrane-associated actin filaments also takes place during mitosis and cell division. For example, in cells grown in monolayer culture, cytoskeletal actin filament bundles (stress fibers) disassemble and dissociate from adhesion sites at the plasma membrane while numerous actin filament–containing microvilli and filopodia (retraction fibers) appear on the surface of the cell when the cells round up in prophase (Erickson and Trinkaus, 1976; Porter et al., 1973, 1974; Sanger and Sanger, 1980; Sanger et al., 1984, 1989). In anaphase, cortical actin filaments are reorganized and recruited to the cleavage furrow to form the contractile ring for cytokinesis (Cao and

Wang, 1990; for review see Salmon, 1989). Finally, after cytokinesis, the contractile ring disassembles, microvilli disappear, and actin filament bundles reappear and reattach to the plasma membrane at sites of cell–cell and cell–substratum attachment. The components and mechanisms regulating cortical actin filament reorganizations and actin filament–membrane associations in mitosis and cell division are not well understood.

Spectrins are a family of long, flexible actin cross-linking proteins which mediate the attachment of actin filaments to the plasma membrane and were originally described in human erythrocytes. The assembly of spectrin with actin filaments, the membrane, and other associated proteins is referred to as the spectrin-based membrane skeleton. Spectrins bind directly or indirectly (via ankyrins) to diverse plasma membrane proteins, including ion pumps, channels, and adhesion receptors, and are believed to play a role in segregating them into distinct domains on the plasma membrane, presumably as a consequence of spectrins' ability to cross-link actin filaments into networks (for reviews see Bennett, 1990a,b; Coleman et al., 1989; Morrow, 1989). In erythrocytes, spectrin functions to regulate membrane shape and stability, as indicated by analysis of spectrin and ankyrin mutations in abnormal erythrocytes from patients with hereditary hemolytic anemias (for review see Davies and Lux, 1989).

The organization and functions of the spectrin-based membrane skeleton have been best characterized in the context of postmitotic, highly differentiated cells, where the spectrin membrane skeleton assumes a fairly stable, restricted distribution characteristic of the phenotype of the particular differentiated cell type. However, recent results indicate that the localization and associations of spectrin can be highly dynamic in mitotic cells. In the rapidly dividing cells of early sea urchin and *Drosophila* embryos, a limited pool of maternal and spectrin appears to translocate between intracellular cytoplasmic vesicles and/or the cytosol to the plasma membrane, redistributing between cleavage furrows and cell–cell contact regions (Schatten et al., 1986; Fishkind et al., 1990b; Pesacreta et al., 1989). Spectrin is restricted to the plasma membranes of postmitotic, polarized, and nonpolarized epithelial cells; however, immunocytochemical localization studies reveal an increased cytoplasmic pool of spectrin in proliferating cells in the basal layer of the epidermis (Yoneda et al., 1990) and in proliferating neoplastic epithelial cells in vivo, including colon adenomas, adenocarcinoma, and ductal carcinoma (Younes et al., 1989). The subcellular distribution of spectrin is also dynamically regulated in activatable cells, for example, after cAMP- and Ca^{2+}-stimulated exocytosis in parotid acinar cells (Perrin et al., 1992), during T lymphocyte activation through the T cell receptor (Lee et al., 1988), and after EGF stimulation of epidermal A431 cells (Bretscher, 1989).

These observations and the molecular properties of spectrin argue that it may play a role in the restructuring of cortical actin filament networks and transformation of cell surface properties that take place during mitosis. As a first approach, we examined the localization and solubility of spectrin during interphase and mitosis in CHO cells and in HeLa cells. We found, by morphological and biochemical approaches, that spectrin redistributes from an insoluble, membrane-associated fraction to a soluble, cytosolic pool in mitosis and undergoes a site-specific increase in phosphorylation on the

β subunit. We suggest that phosphorylation of spectrin in mitosis may reduce spectrin's interaction with cortical actin filaments and with ankyrin (or other membrane binding sites), thus leading to disassembly of the spectrin-based membrane skeleton. Disassembly of the spectrin-based membrane skeleton could be functionally coupled to formation of microvilli and restructuring of membrane-associated actin filament networks, cessation of membrane trafficking, or alterations in cell–cell and/or cell–matrix contacts in mitotic cells.

Materials and Methods

Cell Culture

CHO cells were obtained from L. Gerace (The Scripps Research Institute, La Jolla, CA), HeLa JW36 cells from G. Dreyfuss (University of Pennsylvania, Philadelphia, PA), and HeLa S3 cells from T. Kelly (Johns Hopkins University, Baltimore, MD). CHO cells were grown in monolayer or spinner culture in Joklik's MEM (Gibco Laboratories, Grand Island, NY) containing 10% FCS (HyClone Laboratories, Logan, UT), nonessential amino acids, 0.01 M Hepes buffer, 100 μg/ml penicillin, and 100 μg/ml streptomycin. HeLa JW36 cells were grown in monolayer culture in Joklik's MEM containing 10% enriched calf serum (Gemini BioProducts, Inc., Calabasas, CA), 2.5 g/liter glucose (additional), 0.01 M Hepes, 100 μg/ml penicillin, and 100 μg/ml streptomycin. HeLa S3 cells were grown in spinner culture in Joklik's MEM containing 10% equine serum (HyClone Laboratories) with the same additional components as for the HeLa JW36 cells.

Production and Affinity Purification of Antibodies to Spectrin

Spectrin was purified from bovine brain membranes as described by Bennett et al. (1986), and antibodies were generated in rabbits by injection of spectrin isolated by preparative SDS gel electrophoresis on 7.5% acrylamide gels (Dreyfuss et al., 1984). Antispectrin antibodies were affinity purified over a column of bovine brain spectrin coupled to cyanogen bromide-activated Sepharose (Cl) 4B (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) and eluted with 0.2 M glycine-HCl, pH 2.8, using standard procedures. Preimmune IgG was purified from preimmune serum by affinity chromatography on protein A-Agarose (Pierce Chemical Co., Rockford, IL).

Cell Synchrony and Radiolabeling

To label spectrin to steady state with [^{35}S]methionine, CHO cells were grown to ~50% confluence and radiolabeled for one and one-half doubling times (18–20 h) with 5 μCi/ml [^{35}S]methionine (Trans[^{35}S]label, ICN Radiochemicals, Costa Mesa, CA) in complete Joklik's medium containing 50% of the normal amount of methionine (^{35}S-labeling medium). Dishes or flasks were washed twice in cold Hepes-buffered saline (150 mM NaCl, 1 mM MgCl$_2$, 10 mM Hepes, pH 7.4) and held on ice until extraction (see below). In some experiments, cells were harvested from dishes by brief (1.5–2 min) treatment with trypsin-EDTA, washed twice in cold Hepes-buffered saline by centrifugation and resuspension, and held on ice until extraction. Mitotic CHO cells were isolated as described by Burke and Gerace (1986) and Zieve et al. (1980), with modifications for [^{35}S]methionine labeling. Briefly, cells were subjected to an 11-h thymidine block in the ^{35}S-labeling medium to accumulate cells in early-to-mid S phase, followed by 4–5 h in ^{35}S-labeling medium in the absence of thymidine to allow cells to proceed into G2 phase. After removal of loosely attached cells by a mechanical preshake, 0.1 μg/ml nocodazole was added to the labeling medium to arrest cells in mitosis and cells were collected by mechanical shake-off at 2-h intervals (Zieve et al., 1980). Mitotic cells were washed twice in cold Hepes-buffered saline to remove nocodazole and held on ice to prevent progress through the cell cycle. Using this protocol, 3–4 × 10^6 cells/T175 flask were obtained at the second collection, 90–95% of which were in mitosis, as determined by aceto-orcein staining.

HeLa JW36 cells were grown to ~50% confluence and radiolabeled for approximately one and one-half doubling times (40–42 h) in Joklik's complete medium containing 5 μCi/ml [^{35}S]methionine. [^{35}S]methionine-labeled mitotic HeLa JW36 cells were isolated by mechanical shake-off from cultures which had been subjected to a 24-h thymidine block in com-

plete medium containing 5 μCi/ml [35S]methionine followed by 18–20 h in 0.1 μg/ml nocodazole and 5 μCi/ml [35S]methionine. About 1 × 10^7 cells/T175 flask were obtained and 94–98% of them were in mitosis as determined by aceto-orcein staining.

[35S]Methionine-labeled mitotic HeLa S3 cells were isolated from spinner cultures by a double thymidine block followed by nocodazole treatment (Rao and Johnson, 1970). Briefly, cells were seeded at 2–2.5 × 10^5 cells/ml, grown for 7 h, blocked in thymidine for 18 h, washed, resuspended to 2.5 × 10^5 cells/ml, and returned to culture for 10 h in fresh medium. For the second thymidine block, cells were harvested by centrifugation and resuspended to 2.5 × 10^5 cells/ml in complete medium containing 2 μCi/ml [35S]methionine. After 18 h, cells were washed, resuspended to 2.5 × 10^5 cells/ml, and returned to culture for 4 h in the presence of 2 μCi/ml [35S]methionine. 0.1 μg/ml nocodazole was then added and cells were harvested after 13 h. To obtain 35S-labeled interphase spinner cells, a parallel flask of cells was radiolabeled with 2 μCi/ml [35S]methionine in complete medium for an equivalent period of time (33 h), readjusting the cell density to 2.5 × 10^5 cells/ml 12 h after addition of label.

To label spectrin to steady state with 32P, CHO cells were labeled for 10 h in 50 μCi/ml [32P]orthophosphate in phosphate-free Joklik's medium to which 0.5 mM sodium phosphate had been added (32P-labeling medium). To isolate mitotic CHO cells from cultures labeled with 32P to steady state, cells were subjected to a thymidine block in complete medium as described above; then, the medium was replaced with 50 μCi/ml [32P]-orthophosphate in 32P-labeling medium. After 6 h, loosely attached cells were removed by a preshake, 0.1 μg/ml nocodazole was added, and mitotic cells were harvested at 2-h intervals, corresponding to 8 and 10 h in the 32P-labeling medium (the cells from the 8-h collection were discarded). Asynchronous cultures of HeLa JW36 cells were labeled to steady state with 32P by growth for 17–18 h in 50 μCi/ml [32P]orthophosphate in 32P labeling medium. 32P-labeled mitotic HeLa JW36 cells were obtained from synchronized cultures subjected to a thymidine block as described above, followed by labeling for 17 h with 50 μCi/ml [32P]orthophosphate in the presence of 0.1 μg/ml nocodazole. For tryptic phosphopeptide mapping and phosphoamino acid analysis of spectrin, CHO cells were labeled for only 1 h with 1 mCi/ml [32P]orthophosphate in phosphate-free Joklik's medium. Mitotic cells were obtained by mechanical shake-off from synchronized cultures after a 1-h accumulation in phosphate-free Joklik's medium containing 1 mCi/ml [32P]orthophosphate and 0.1 μg/ml nocodazole. 32P-labeled G2-enriched cells were obtained from the cells that remained attached to the flask.

Extraction of Cells

Whole Cell Extracts. Total cell extracts were prepared for immunoprecipitation by solubilization of whole cells (either in situ on dishes or as cell pellets) in a nondenaturing, Triton–high salt buffer at pH 8.8 (TX/HSB/pH 8.8), containing 1% Triton X-100, 0.5 M NaCl, 2 mM EDTA, 20 mM NaF, 2.5 mM EGTA, 10 mM sodium pyrophosphate, 20 mM Tris-HCl, pH 8.8, and the following protease inhibitors: 1 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, 100 μg/ml tosyl-L-lysine chloromethyl ketone, and 100 μg/ml PMSF. Cells were solubilized at a concentration of 2 × 10^6 cells/ml. After sonication with a microprobe sonicator (50–100 pulses, 30% output, setting 8 [Heat-Systems Ultrasonics, Plainsview, NY]), extracts were centrifuged for 20 min at 100,000 g in a TLA100.3 rotor (Beckman Instrs. Inc., Fullerton, CA) to remove residual insoluble material. To prepare samples for immunoprecipitation, an equal volume of a Triton–high salt buffer at pH 6.8 (TX/HSB/pH 6.8) (containing 20 mM Hepes-KOH at pH 6.8 instead of 20 mM Tris-HCl at pH 8.8) was added to bring the final pH of the extracts to pH 7.3–7.5, and the cell concentration to 10^6 cell equivalents/ml (ceq/ml). In some experiments, cells (2–3 × 10^6) were solubilized in 0.4% SDS, 0.15 M NaCl, 10 mM sodium phosphate, pH 7.4, 2 mM EDTA containing protease inhibitors, and boiled for 3 min. After sonication, Triton X-100 was added to a final concentration of 2%, iodoacetic acid to 5 mM, and the cell concentration was brought to 10^6 ceq/ml by addition of PBS.

To prepare extracts of 32P-labeled cells, cells were solubilized as described above in TX/HSB/pH 8.8 except that the buffer also contained 1 mM MgCl_2 and only 0.2 mM EDTA. After neutralization by addition of an equal volume of TX/HSB/pH 6.8 buffer (also containing 1 mM MgCl_2 and 0.2 mM EDTA), extracts were treated with 50 μg/ml DNase I and 10 μg/ml RNase A (Boehringer Mannheim Biochemicals, Indianapolis, IN) for 20–30 min at room temperature. To inactivate proteases, stock solutions of

1. *Abbreviations used in this paper:* ceq, cell equivalent; HSB, high salt buffer; LSB, low salt buffer; TX, Triton X-100.

DNase I and RNase A were pretreated with 2.5 mM diisopropylfluorophosphate (Sigma Chemical Co., St. Louis, MO) for 30 min on ice, and RNase A was incubated for an additional 10 min in a boiling water bath. After addition of EDTA to 5 mM and ATP to 1 mM to the extracts, they were added to antispectrin-coated protein A beads and spectrin was immunoprecipitated as described below.

Triton X-100 Extraction. A soluble and a membrane/cytoskeleton fraction was prepared from cells by extraction with Triton X-100 in a low salt buffer (TX/LSB) containing 0.5% Triton X-100, 10 mM K acetate, 2.5 mM MgCl_2, 5 mM EGTA, and 10 mM Hepes-KOH, pH 7.4. This and all other extraction solutions contained the protease inhibitors listed above. Cells growing on dishes were extracted in TX/LSB for 5–10 min at 4°C on a rocking platform at a concentration of 4 × 10^6 cells/ml (determined by counting cells harvested by trypsin-EDTA from a duplicate dish); then dishes were tilted to collect the soluble fraction. The insoluble material remaining on the dish (containing the membrane/cytoskeletons) was solubilized by scraping it off the dish into enough TX/HSB/pH 8.8 to bring it to 2 × 10^6 ceq/ml, sonicated extensively as above, and then centrifuged for 20 min at 100,000 g in the TLA100.3 rotor (Beckman Instrs., Inc.) at 4°C to remove residual insoluble material. An equal volume of 2× strength TX/HSB/pH 8.8 was added to the soluble fraction to bring it also to 2 × 10^6 ceq/ml. For cells harvested by brief trypsin-EDTA, as well as for mitotic cells and spinner cells, washed cells were resuspended gently by pipetting up and down in TX/LSB at 4 × 10^6 ceq/ml, incubated for 30 min at 4°C, and then the Triton-insoluble material was collected by centrifugation for 20 min at 100,000 g in the TLA100.3 rotor at 4°C. The pellet was solubilized as above by extensive sonication in TX/HSB/pH 8.8 and centrifugation to remove residual insoluble material. Further extraction of this residual material with boiling SDS demonstrated that this procedure solubilized all of the spectrin from the TX/LSB insoluble fraction (not shown). Finally, an equal volume of TX/HSB/pH 6.8 was then added to each fraction to bring the final pH of the extracts to 7.3–7.5, and the cell equivalent to 10^6 ceq/ml.

Immunoprecipitation and SDS Gel Electrophoresis

Extracts from [35S]methionine or 32P-labeled cells were prepared as described above and incubated overnight at 4°C on an end-over-end rotator with affinity-purified, anti-bovine bain spectrin antibodies (2 μg/10^6 ceq) which had been preadsorbed to protein A-Trisacryl beads (Pierce Chemical Co., Rockford, IL). This amount of antibody was sufficient to quantitatively immunoprecipitate >90% of the spectrin, as determined by sequential immunoprecipitations performed on the same extract. Beads were then washed four times with a 1:1 mixture of TX/HSB/pH 8.8:TX/HSB/pH 6.8 (final pH 7.3–7.5) containing 1 mg/ml BSA (Fraction V [ICN Biomedicals, Costa Mesa, CA]), followed by one wash with the same buffer without BSA, and two washes with 10 mM Tris-HCl, pH 7.4. Finally, spectrin was eluted from the immunoadsorbent beads in SDS gel sample buffer and electrophoresed on 7.5% acrylamide gels with a 3% stacking gel according to the method of Dreyfuss et al. (1984). After electrophoresis, gels containing [35S]methionine-labeled proteins were stained in Coomassie blue, prepared for fluorography with EN3HANCE (New England Nuclear, Boston, MA), dried, and exposed to preflashed x-ray film (XAR-5, Eastman Kodak Co., Rochester, NY) at −70°C (Laskey, 1980). The amounts of 35S-labeled spectrin polypeptides in each lane were determined by quantitative densitometry with scanning densitometer (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). In the experiments presented, the percent of spectrin in the soluble and insoluble fraction is expressed as a percent of the spectrin recovered from both fractions combined. In all cases, total recoveries were within 80–120% of the spectrin immunoprecipitated from an equivalent amount of whole cell extract.

Phosphopeptide Mapping and Phosphoamino Acid Analysis

After immunoprecipitation and SDS-PAGE, spectrin polypeptides were localized by staining with Coomassie blue, cut from the gel, and subjected to a limit trypsin digestion (Ottaviano and Gerace, 1985). For phosphopeptide mapping, the tryptic digest was lyophilized and analyzed by electrophoresis and chromatography on cellulose thin-layer plates as described (Elder et al., 1977) with modifications (Hubbard and Ma, 1983). For phosphoamino acid analysis, after digestion and lyophilization, digests were partially hydrolyzed in 6 N HCl and separated on cellulose thin-layer plates by electrophoresis followed by chromatography (Ottaviano and Gerace, 1985; Hunter and Sefton, 1980). The migration positions of phosphoserine, phosphothreonine, and phosphotyrosine were obtained by inclusion of 1 μg

of nonradioactive phosphoamino acid standards (Sigma Chemical Co., St. Louis, MO) in each sample.

Immunofluorescence Microscopy

CHO cells were grown on glass coverslips in Joklik's complete medium and fixed for 15 min at room temperature in 4% formaldehyde (ultrapure, methanol free [Polysciences, Inc., Warrington, PA]) in Dulbecco's PBS. After permeabilization with 0.1% Triton X-100 in PBS, coverslips were labeled for 1 h at room temperature with 10 μg/ml affinity-purified antispectrin or preimmune IgG followed by a 1:200 dilution of fluorescein or rhodamine-conjugated goat anti-rabbit IgG (Cappell Worthington Biochemicals, Malvern, PA) for 45 min at room temperature. In some experiments, F-actin was stained with a 1:400 dilution of rhodamine phalloidin (0.0825 nM; Molecular Probes, Inc., Eugene, OR) for 45 min at room temperature and DNA was stained with 1 μg/ml Hoechst dye No. 33258 (Calbiochem Corp., La Jolla, CA) for 3 min at room temperature. Coverslips were mounted on slides over a drop of 40 mM Tris-HCl, pH 8.0, 75% glycerol, 0.1% p-phenylenediamine to prevent bleaching, and sealed with nail polish. Rhodamine and fluorescein staining was observed with a 63× oil immersion Planapo objective and Hoechst staining was observed with a 40× Neofluor objective on a Zeiss Axiophot, using the appropriate barrier filters. Micrographs were taken with the Zeiss automatic camera set to 400 ASA using T-MAX film (Eastman Kodak Co., Rochester, NY).

Results

Identification of Spectrin in CHO Cells

We prepared polyclonal antibodies to the major α subunit isoform of spectrin (often referred to as α-fodrin) by using bovine brain spectrin as an immunogen. These antibodies specifically immunoprecipitate a polypeptide from CHO cells that comigrates with the α subunit of bovine brain spectrin on SDS–polyacrylamide gels when cells are solubilized in SDS (Fig. 1, compare lane c with lane a). When CHO cells are solubilized in a nondenaturing, high salt buffer containing Triton X-100, equimolar amounts of a polypeptide comigrating with the β subunit of brain spectrin are coimmunoprecipitated (Fig. 1, lane d). Since spectrin consists of equimolar amounts of α and β subunits, this strongly indicates that these antibodies are specific for the α subunit of CHO cell spectrin.

Immunofluorescence Localization of Spectrin in Interphase and Mitotic CHO Cells

Immunofluorescence staining of substrate-attached, well-spread interphase CHO cells reveals a fibrillar pattern of spectrin staining (Fig. 2). This fibrillar staining is predominantly associated with both the dorsal and ventral plasma membranes of the cells, as determined by adjusting the focal plane, and is excluded from the actin-containing stress fibers on the ventral plasma membrane (not shown; see Levine and Willard, 1981; Mangeat and Burridge, 1984). In areas of cell–cell contact, there is an increased concentration of spectrin staining at the plasma membrane (Fig. 2 A, arrows). In some cells, bright staining of ruffling membranes indicates that spectrin staining is also associated with the plasma membrane in these regions (Fig. 2 C, arrows). However, spectrin staining is not present in small actin-containing microspikes which are occasionally visible at the leading edge of cells (not shown). Preimmune IgG does not stain the cells at all (Fig. 2 E). This staining pattern is similar to previous descriptions of plasma membrane staining for spectrin in various cultured cell types (Burridge et al., 1982; Le-
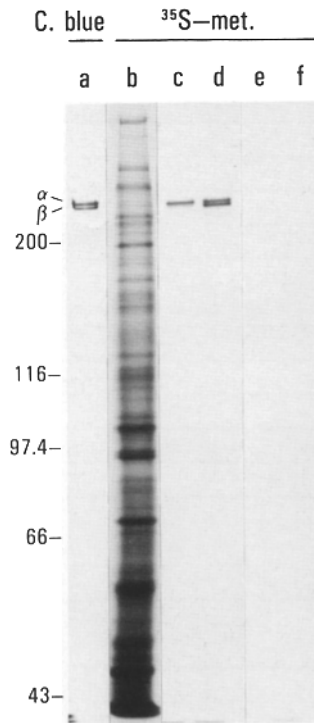

Figure 1. Identification of spectrin α and β polypeptides in CHO cells. Coomassie blue-stained gel of purified bovine brain spectrin (2 μg) (lane a), [^{35}S]methionine-labeled CHO cell proteins (lane b), [^{35}S]-methionine-labeled spectrin polypeptides immunoprecipitated with anti-bovine brain spectrin antibodies from CHO cells which had been solubilized with SDS (lane c) or with TX/HSB/pH 8.8 (lane d), and [^{35}S]methionine-labeled proteins nonspecifically immunoprecipitated with preimmune IgG from SDS (lane e) or Triton–high salt extracts (lane f). CHO cells were labeled to steady state in medium containing [^{35}S]methionine, and spectrin was immunoprecipitated from cell extracts prepared by solubilization in SDS followed by addition of excess Triton X-100 (lanes c and e), or by solubilization in nondenaturing TX/HSB/pH 8.8 (lanes d and f) (see Materials and Methods). Samples were electrophoresed on 7.5% SDS gels and processed for fluorography to detect [^{35}S]methionine-labeled polypeptides in lanes b–f. The antispectrin antibodies are specific for the α subunit since only the α subunit is immunoprecipitated from extracts solubilized in SDS (lane c), while an equimolar complex of the spectrin α, β heterodimer can be immunoprecipitated from extracts solubilized in Triton–high salt under nondenaturing conditions (lane d). Note, using this gel system (Dreyfuss et al., 1984), the apparent mobilities of the spectrin α and β polypeptides are M_r 225,000 and 220,000, respectively, instead of the reported values of M_r 240,000 and 235,000 (Bennett, 1990b).

vine and Willard, 1981; Mangeat and Burridge, 1984; Kaiser et al., 1989).

Mitotic CHO cells are small, round cells that are covered with many small, actin-containing microvilli (3–4 μm long) and are anchored to the substrate by long, actin-containing filopodia (also termed retraction fibers) that extend from the cell body to the substrate and can be up to 10–15 μm long (Erickson and Trinkaus, 1976; Porter et al., 1973). Immunofluorescence staining for spectrin in mitotic CHO cells reveals a predominantly cytoplasmic localization, with only very faint staining on the plasma membrane over the cell body (Figs. 3 and 4), and no discernible staining in the actin filament-containing microvilli and filopodia that stain brightly with rhodamine phalloidin (Fig. 4). In favorable images of large metaphase cells, the faint spectrin staining on the plasma membrane over the cell body appears to be somewhat discontinuous (Fig. 3). The areas without spectrin staining may be due to filopodia extending from these locations on the plasma membrane. No differences in spectrin staining were observed at different stages of mitosis; e.g., compare the anaphase cell marked with an asterisk in the bottom panel of Fig. 4 with the metaphase cells in Figs. 3
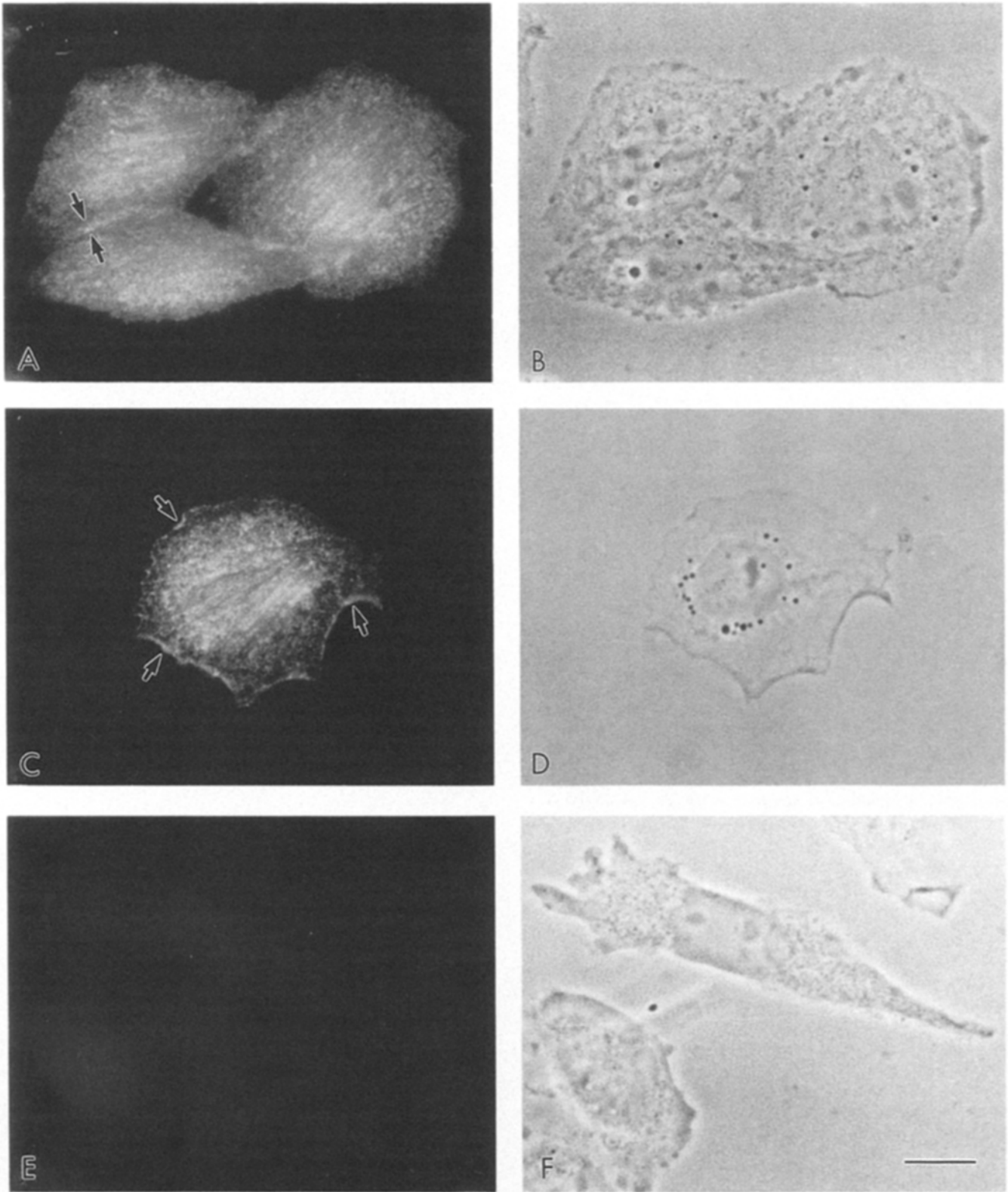
Figure 2. Immunofluorescence staining of attached, interphase CHO cells with antispectrin (*A* and *C*), or with preimmune IgG (*E*). *B*, *D*, and *F* are phase-contrast micrographs corresponding to *A*, *C*, and *E* respectively. Spectrin staining appears fibrillar on both dorsal and ventral plasma membrane surfaces and is associated with regions of cell–cell contact (*A*, *arrows*) as well as with ruffling membranes (*B*). The focal plane of the phase micrograph in *D* is shifted slightly compared to that of the companion fluorescence micrograph in *C*. Bar, 12 μm.
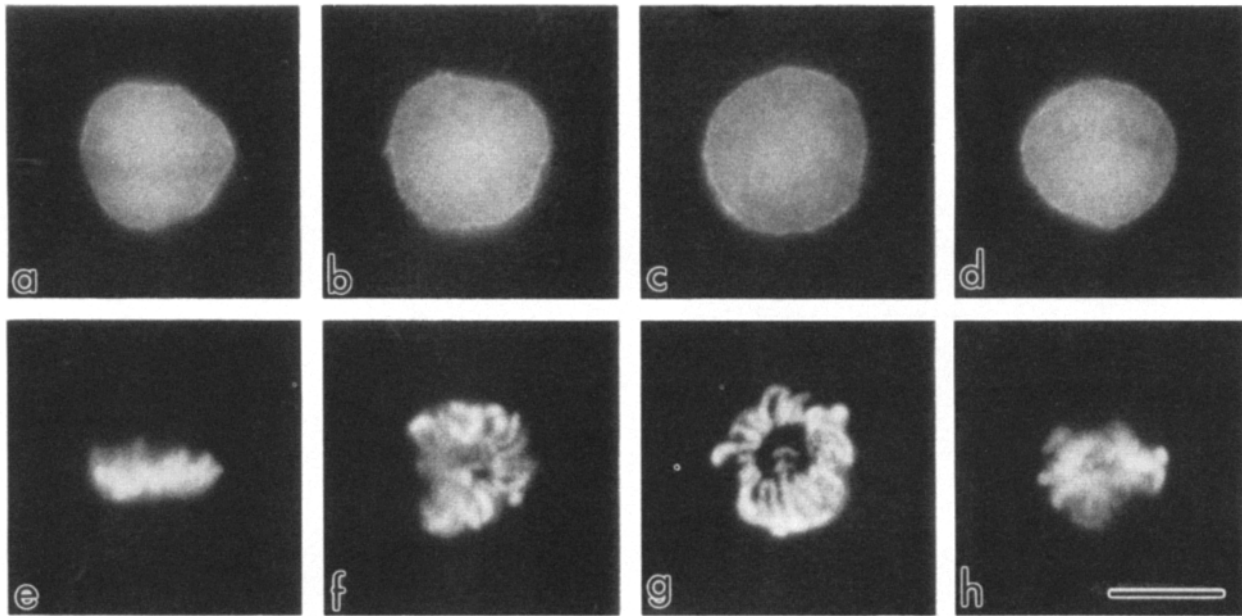
Figure 3. Staining of metaphase CHO cells with antispectrin antibodies (*a–d*) and with Hoechst dye to localize the chromosomes (*e–h*). Spectrin staining is predominantly in the cytoplasm, with only faint staining on the plasma membrane at the periphery of the cell body. Note that these cells were photographed in a variety of different orientations with respect to the metaphase plate. The mottled appearance of the cytoplasmic staining for spectrin in some of the cells is probably due to exclusion of spectrin from areas of cytoplasm occupied by chromosomes (compare spectrin staining with HOECHST staining). To enrich for mitotic cells, CHO cells growing on coverslips were subjected to an 11-h thymidine block followed by a 5-h recovery period in Joklik's complete medium without thymidine. Bar, 12 μm.

and 4. Also, spectrin staining was not concentrated in the cleavage furrow (not shown), unlike dividing cells in early mouse (Schatten et al., 1986), sea urchin (Fishkind et al., 1990b), or *Drosophila* embryos (Pesacreta et al., 1989). After telophase, when the two daughter cells enter the G1 phase of the cell cycle and begin to spread out on the culture dish, fibrillar spectrin staining associated with the dorsal and ventral plasma membranes reappears (not shown).

These observations are consistent with the possibility that spectrin dissociates from the plasma membrane and redistributes to the cytoplasm when CHO cells round up in mitosis. The diffuse appearance of the cytoplasmic spectrin staining in mitotic cells further suggests that much of the spectrin in mitotic cells may be cytosolic. However, it is not possible to determine from immunofluorescence staining and conventional light microscopy what the relative proportions of plasma membrane–associated and cytosolic spectrin are in interphase and mitotic cells. For example, it is possible that some spectrin may be associated with cytoplasmic membranes and/or internal cytoskeletal components that are not clearly resolved from the dorsal and ventral plasma membranes in the flattened interphase cells, and/or are below the resolution of the light microscope. Therefore, we have used a biochemical approach to determine the amount of soluble spectrin in interphase and mitotic cells.

Spectrin Solubility in Interphase and Mitotic CHO Cells

As a first approach, we determined the amount of soluble spectrin obtained after Triton X-100 extraction of flat interphase cells attached to tissue culture plastic and of synchronized populations of round mitotic cells. This approach

has been used previously to examine spectrin solubility and association with the membrane skeleton in differentiating erythroblasts (Lazarides and Woods, 1989) and in polarized epithelial cells in culture (Rodriguez-Boulan and Nelson, 1989). It is important to note that the Triton-insoluble residue of nucleated cells is expected to include components from the internal cytoskeleton as well as from the plasma membrane skeleton.

We measured the amounts of Triton-soluble and -insoluble spectrin by quantitatively immunoprecipitating spectrin from Triton X-100 extracts of CHO cells that had been radiolabeled to steady state with [^{35}S]methionine for 18–20 h. Spectrin in the Triton-insoluble residue was solubilized in preparation for immunoprecipitation by reextraction with TX/HSB/pH 8.8 (see Materials and Methods). After immunoprecipitation, samples were electrophoresed on SDS gels and the amount of [^{35}S]methionine-labeled spectrin in each sample was determined by laser scanning densitometry of the gels after fluorography. In separate experiments, we determined that 18–20 h was sufficient to label both Triton-soluble and -insoluble pools of CHO spectrin to steady state (not shown).

In attached, asynchronous populations of interphase CHO cells ~85% of the spectrin is Triton-insoluble and only ~15% is Triton-soluble. In contrast, in nocodazole-arrested, mitotic CHO cells isolated by mechanical shake-off, 60% of the CHO cell spectrin is Triton-soluble (Fig. 5). This represents a fourfold increase in the percentage of soluble spectrin as compared to interphase cells. This increase in the amount of soluble spectrin is mitosis specific since populations of G2-enriched and G1 cells have a distribution of Triton-soluble and -insoluble spectrin characteristic of the average interphase state (Table I). These results also demon-
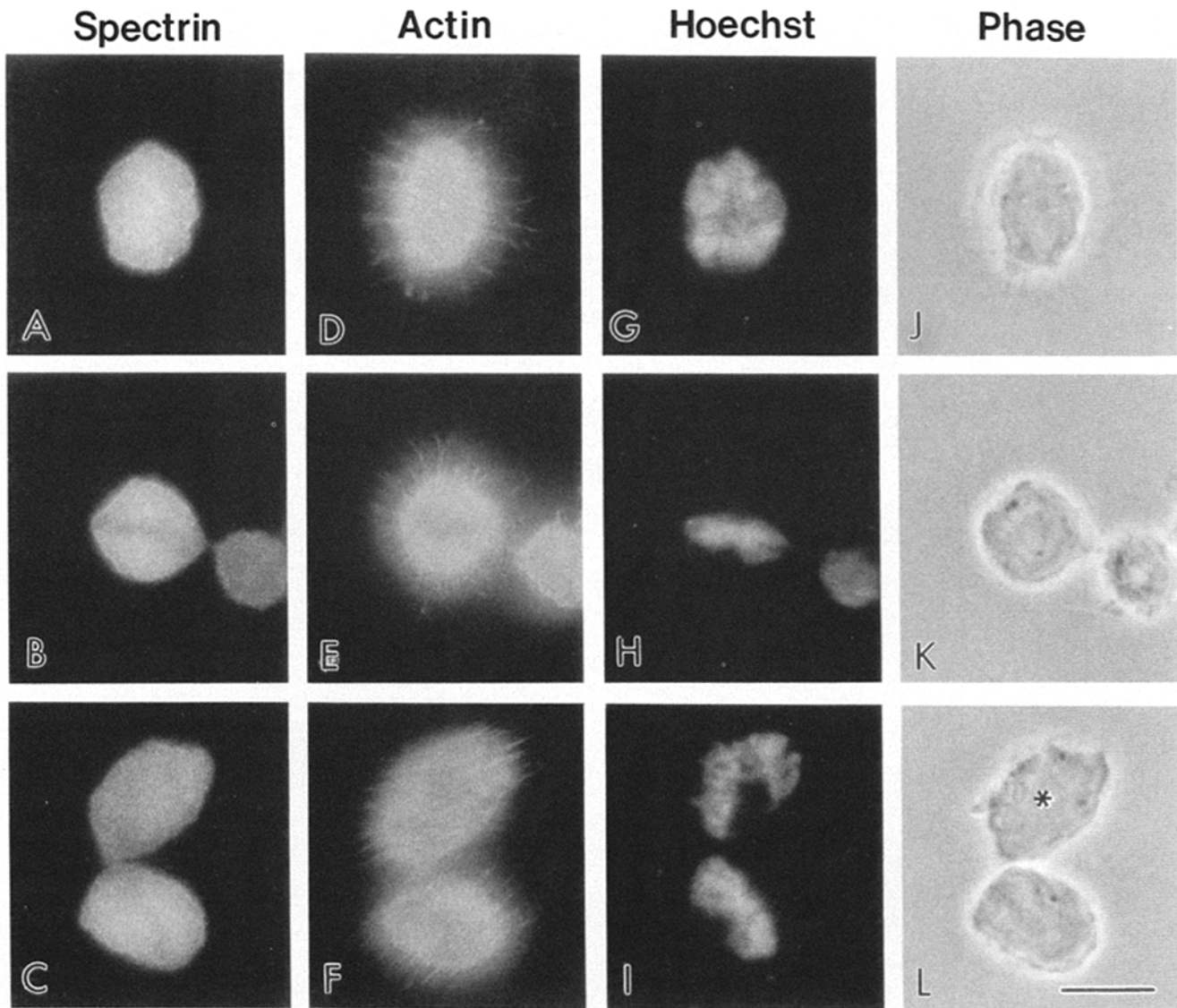
Spectrin	Actin	Hoechst	Phase

Figure 4. Staining of mitotic CHO cells with antispectrin antibodies (*A–C*), rhodamine phalloidin to localize F-actin (*D–F*), and with HOECHST dye to localize the chromosomes (*G–I*). *J–L* are phase-contrast micrographs corresponding to the cells in the top, middle, and bottom panels, respectively. As in Fig. 3, spectrin staining is predominantly cytoplasmic and is not discernible in the numerous rhodamine phalloidin-staining microvilli and filopodia (retraction fibers) that cover the surface of the mitotic cells. Rhodamine phalloidin also stains very long filopodia that are two to three times as long as the diameter of the cell body, but this staining is difficult to reproduce photographically. Except occasionally, most of the filopodia are not visible in phase-contrast microscopy (see cell in top panels). Note that the distribution of spectrin does not appear to be different in anaphase cells (cell marked with asterisk in *L* and possibly cell in *J*) as compared with metaphase cells (remaining cells in figure; also see Fig. 3). Mitotic cells were enriched as described in the legend to Fig. 3. Bar, 12 µm.

strate that the increase in Triton-soluble spectrin in mitosis is not an artifact of nocodazole treatment, since the G2-enriched cells were obtained from the attached cells remaining on the dishes after collection of the nocodazole-arrested mitotic cells; thus, the G2-enriched cells had been exposed to nocodazole for the same amount of time as the mitotic cells.

The total amount of [^{35}S]methionine-labeled spectrin immunoprecipitated per mitotic cell is the same as that immunoprecipitated per average interphase or G2 cell, and is approximately twice the amount immunoprecipitated per G1 cell (Fig. 5 *A* and data not shown). This indicates that the increased percentage of Triton-soluble spectrin in mitotic cells does not result from new synthesis of soluble spectrin or a selective degradation of insoluble spectrin, and also that the soluble spectrin in mitotic cells is not preferentially degraded when the cells divide and enter G1. However, it is important to point out that our experiments measure the total amount of spectrin in soluble and insoluble pools, and provide no direct information concerning the biosynthesis and turnover of spectrin in soluble and insoluble pools during the cell cycle.

Microinjection of calpain into cultured cells has been reported to promote the onset of metaphase (Schollmeyer, 1988). The above experiments indicate that calpain cleavage of spectrin does not occur in mitotic cells. In addition, we

A

	Total	Insol.	Sol.
	a a	b b	c c

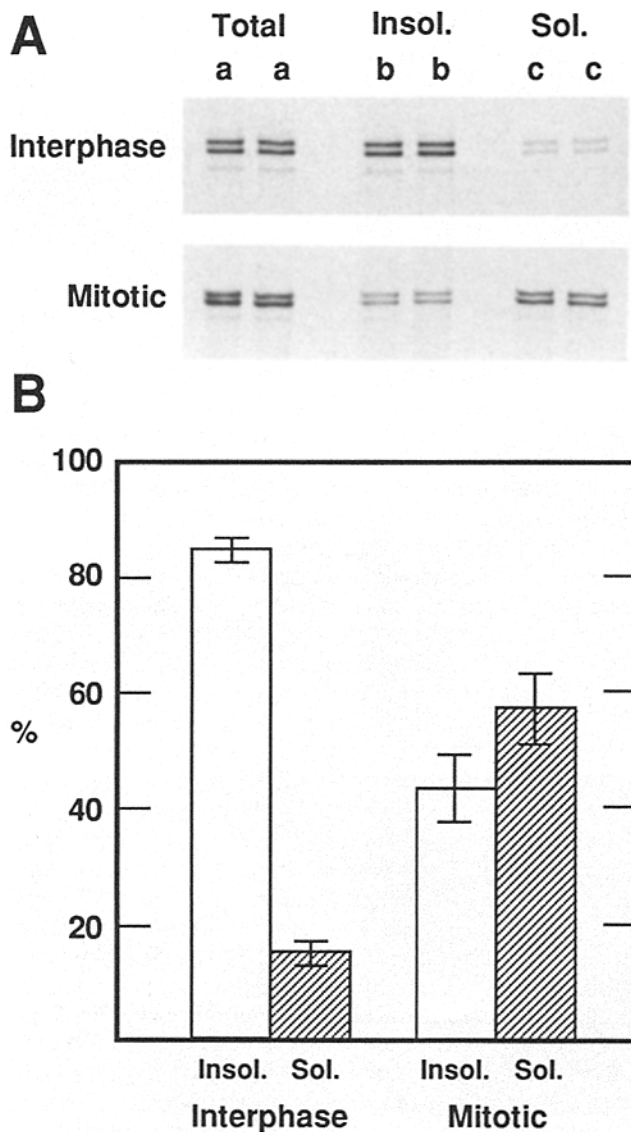Interphase

Mitotic

B

Figure 5. Analysis of the solubility properties of spectrin in interphase CHO cells growing attached to plastic and in mitotic CHO cells isolated by mechanical shake-off from attached cultures. (A) Fluorograms of [35S]methionine-labeled spectrin immunoprecipitated from duplicate samples of whole cell extracts (Total), the Triton-insoluble fraction (Insol.), and the Triton-soluble (Sol.) fraction of asynchronous interphase cells attached to plastic, and nocodazole-arrested mitotic cells isolated by mechanical shake-off from attached cultures (10^6 cells/sample). (B) Percent of spectrin in Triton-soluble and -insoluble fractions from interphase and mitotic cells. Graph represents the average of results from 5 separate experiments for the interphase cells and 11 separate experiments for the mitotic cells, and the error bars are the standard deviations.

did not observe the characteristic calpain cleavage products of spectrin at 120–165 kD, either in CHO (Fig. 6) or in HeLa cells (not shown) (Harris and Morrow, 1990; Hu and Bennett, 1991).

Relationship of Spectrin Solubilization to Cell Detachment in Mitosis

To investigate whether the increased percentage of Triton-soluble spectrin in mitotic cells could be due to cell de-

Table I. Spectrin Solubility in CHO Cells at Different Stages in the Cell Cycle

Cell cycle stage	Insoluble	Soluble	n
	%	%	
I	85.0	15.0	5
G1	84.4	15.6	2
G2	86.8	13.2	2
M	43.0	57.0	11

Spectrin was immunoprecipitated from the Triton-soluble or -insoluble fraction of [35S]methionine-labeled cultures of asynchronous interphase CHO cells growing attached to plastic (I), mitotic cells (M) isolated by mechanical shake-off from attached cultures, or G2-enriched populations of cells (G2) remaining after collection of mitotic cells. G1 cells were obtained by returning mitotically selected cells to culture for 90 min and then washing off any nonadherent cells. At this time >90% of the cells attached to the dishes had completed mitosis, as determined by aceto-orcein staining. n = number of experiments.

tachment from the substratum and/or from other cells during mitosis, we compared spectrin solubility in cells grown attached to tissue culture plastic with spectrin solubility in cells detached from dishes by trypsin-EDTA, and in cells grown in suspension in spinner culture. For these experiments, we used CHO cells and two strains of HeLa cells. CHO cells can grow either in spinner culture or in monolayer culture attached to plastic. HeLa JW36 cells are a strain of HeLa cells that grow only in monolayer culture on tissue culture plastic and were originally isolated based on their tightly adherent phenotype. Both CHO and HeLa JW36 cells are epithelial-like and tend to grow associated in clusters with a well-spread, polygonal morphology when grown attached to tissue culture plastic (Fig. 2 A and data not shown). The

Table II. Effect of Cell Attachment and Mitosis on Spectrin Solubility

Cells	Cell cycle stage	Conditions	Insoluble	Soluble	n
			%	%	
A. CHO	I	Attached	85.0	15.0	5
	I	Trypsin-EDTA	66.5	33.5	20
	I	Spinner	61.7	38.3	2
	M	Shake-off	43.0	57.0	11
B. HeLa JW36	I	Attached	89.0	11.0	3
	I	Trypsin-EDTA	62.9	37.2	2
	M	Shake-off	38.8	61.2	5
C. HeLa S3	I	Spinner	75.3	24.7	3
	M	Spinner	40.9	59.1	4

Spectrin was immunoprecipitated from the Triton-soluble or -insoluble fraction of 35S-methionine-labeled, asynchronous cultures of interphase (I) cells growing attached in monolayer culture or in spinner culture, or from mitotic (M) cells obtained as described in Materials and Methods. Interphase cells growing in monolayer culture were either extracted with Triton in situ on the culture dish, or were detached from the dish by trypsin-EDTA before extraction with Triton. The fraction of cells in mitosis obtained from synchronized HeLa S3 spinner cultures varied between 83 and 88% as determined by aceto-orcein staining. Therefore, the percent of spectrin associated with the membrane skeleton was corrected for insoluble spectrin contributed by contaminating interphase cells according to the formula: (fraction of mitotic cells) (χ) + (fraction of interphase cells) (% insoluble spectrin in interphase cells) = % insoluble spectrin in populations of nocodazole-arrested HeLa S3 cells, where χ is the % insoluble spectrin expected for a homogenous population of mitotic HeLa S3 cells. Values for CHO and HeLa JW36 mitotic cells are not corrected since the fraction of cells in mitosis was 94–98% in all experiments. n = number of experiments.

HeLa S3 cells we used grow only in spinner culture and do not attach to plastic.

In asynchronous populations of interphase CHO and HeLa JW36 cells attached to tissue culture plastic, ~85–89% of the spectrin is associated with the Triton-insoluble fraction and only 11–15% of the spectrin is soluble (Table II, A and B). When cells are detached from one another and the substratum by brief treatment with trypsin-EDTA or by growing in suspension in spinner culture, the amount of Triton-soluble spectrin increases to 33–38% in both cell types (Table II). This is about one half the amount of Triton-soluble spectrin that is observed in mitotic cells isolated by mechanical shake-off from attached cultures of either CHO or HeLa JW36 cells (57–60% (Table II, A and B).

Next, we investigated whether the increase in Triton-soluble spectrin in mitotic cells isolated by mechanical shake-off from attached cell cultures might be synergistic with cell detachment–correlated increases in soluble spectrin. For these experiments, we examined spectrin solubility in Triton extracts of interphase and mitotic cells isolated from spinner cultures of HeLa S3 cells, which could be synchronized by a double thymidine block and nocodazole treatment to achieve a population of cells in which ~85% of the cells were arrested in mitosis (Rao and Johnson, 1970). (Spinner cultures of CHO cells could not be synchronized to achieve a sufficiently high percentage of cells in mitosis for biochemical analysis.) In asynchronous interphase populations of HeLa S3 cells, ~75% of the spectrin is associated with the Triton-insoluble fraction and 25% is soluble. In nocodazole-arrested mitotic cells from these HeLa S3 spinner cultures, the percentage of Triton-soluble spectrin increases ~2.5-fold to 60% (Table II, C). This value is equal to that observed for nocodazole-arrested mitotic cells isolated by mechanical shake-off from attached cultures of CHO or HeLa JW36 cells (60%) (Table II, A and B).

These experiments indicate that cell detachment leads to an increase in the amount of soluble spectrin in both CHO and HeLa cells, but not as much of an increase as does entry into mitosis. Redistribution of spectrin from the Triton-insoluble fraction to the soluble fraction during mitosis occurs whether cells are grown attached to plastic or in suspension in spinner culture. However, the effect of mitosis on

spectrin solubility is not additive with the effect of growth in suspension. The maximal percentage of soluble spectrin in mitotic cells is the same (60%) whether mitotic cells are isolated from attached or spinner cultures, indicating that the pool of spectrin molecules susceptible to solubilization may be limited.

The Increase in Triton-soluble Spectrin in Mitosis Reflects Spectrin Dissociation from Membranes

An increase in the proportion of Triton-soluble spectrin in mitotic cell extracts could reflect dissociation of spectrin from actin filaments or other cytoskeletal components without dissociation from membrane-binding site(s). If so, then we would expect that spectrin would remain associated with the membrane fraction after homogenization of mitotic cells in the absence of detergent. To test this possibility, we homogenized interphase and mitotic CHO cells in a ball-bearing homogenizer in the absence of detergent and prepared a cytosol and particulate fraction by centrifugation at 100,000 g (Balch and Rothman, 1985). For this experiment, a suspension of interphase cells suitable for homogenization was obtained by mechanically scraping them from the culture flasks; it is not possible to homogenize cells when they are attached to tissue culture plastic and cells detached by trypsin-EDTA treatment are extremely difficult to homogenize. We found that 30% of the total spectrin was present in the 100,000-g supernatant from homogenates of interphase CHO cells, similar to the percentage of Triton-soluble spectrin observed in CHO cells detached from the substratum and one another by a variety of means, including mechanical scraping (Table II and data not shown). In contrast, 66% of the total spectrin was present in the 100,000-g supernatant from mitotic cell homogenates, similar to the percentage of soluble spectrin in Triton extracts of mitotic cells (Fig. 5 and Table IIA). Therefore, the increased proportion of Triton-soluble spectrin in mitotic cells is likely to reflect dissociation of spectrin from membrane-binding site(s) as well as from actin filaments or other cytoskeletal components.

In addition, soluble spectrin from Triton extracts of mitotic and interphase CHO cells cosediments with purified brain spectrin tetramer on sucrose gradients (data not

Table III. Moles Phosphate/Mole Spectrin Dimer at Steady State in Interphase and Mitosis

Experiment	Cells	Cell cycle stage	Conditions	Mol ^{32}P / Mol spectrin
1	CHO	I	Attached	0.93
		I	Trypsin-EDTA	0.89
2	CHO	I	Trypsin-EDTA	1.18
		G2	Trypsin-EDTA	1.09
		M	Shake-off	1.59
3	HeLa JW36	I	Attached	1.39
		I	Trypsin-EDTA	1.41
		M	Shake-off	2.39

CHO or HeLa JW36 cells were labeled to steady state with [^{32}P]orthophosphate for 10 or 18 h, respectively, and spectrin was immunoprecipitated from 10^7 interphase cells growing attached to plastic (extracted in situ or after detachment by trypsin-EDTA) (I), mitotic cells (M) isolated by mechanical shake-off, or G2-enriched cells remaining after collection of mitotic cells (G2). After electrophoresis on SDS gels and staining with Coomassie blue, the protein mass in the spectrin α and β subunits was determined spectrophotometrically using purified bovine brain spectrin as a standard (Fenner et al., 1975). Subsequently, the moles of phosphate associated with the spectrin α, β dimer were determined by scintillation counting of the gel slices, assuming a molecular weight for the spectrin α, β dimer of 4.75 × 10^5 g/mol, and that the specific activity of the media and the spectrin were at equilibrium.

shown). This further suggests that soluble spectrin in mitotic cells is not associated with ankyrin, small actin oligomers, or any other proteins. This is unlike the situation in MDCK epithelial cells in culture where a fraction of the Triton-soluble, unassembled spectrin in nonpolarized cells is present in a high molecular weight complex with ankyrin and the $Na^+/K^+ATPase$, or with ankyrin and uvomorulin (cadherin) (for review see Rodriguez-Boulan and Nelson, 1989).

Spectrin Phosphorylation in Interphase and Mitotic Cells

Phosphorylation of cytoskeletal components has been shown to induce disassembly of a number of cytoskeletal structures during mitosis, including the nuclear lamina and vimentin-containing intermediate filaments (for reviews see Lewin, 1990; Pines and Hunter, 1990). To determine whether an analogous mechanism could play a role in solubilization of spectrin during mitosis, we compared spectrin phosphorylation in interphase and mitotic cells that had been metabolically labeled with [^{32}P]orthophosphate. To look at mitosis-specific phosphorylation independent of the possible effects of cell rounding and detachment from the substratum, we compared spectrin phosphorylation in mitotic cells isolated by mechanical shake-off to spectrin phosphorylation in cells detached from dishes by brief treatment with trypsin-EDTA. In control experiments (Table III), we determined that the level of spectrin phosphorylation was the same in attached and in trypsin-EDTA-detached interphase cells.

CHO cell spectrin is phosphorylated exclusively on the β subunit in both interphase and mitotic cells. There is an increase of ~40% in the relative amount of ^{32}P incorporated into the spectrin β subunit isolated from mitotic as compared to interphase cells, as determined by scanning densitometry of the autoradiograms (Fig. 6), or by scintillation counting (see Table III). HeLa JW36 cell spectrin is also phosphorylated on the β subunit (not shown), as is spectrin in all cell types examined to date (for reviews see Morrow, 1989; Bennett, 1990a,b). Acid hydrolysis and two-dimensional phosphoamino acid analysis of ^{32}P-labeled β-spectrin isolated from metabolically labeled CHO cells demonstrates that phosphoserine is the major phosphorylated amino acid in both interphase and mitotic cells (Fig. 7). Phosphothreonine is barely detectable and phosphotyrosine is absent, even on longer exposures of the maps.

To quantify the actual moles of phosphate incorporated per mole of spectrin in interphase and mitotic cells, CHO or HeLa JW36 cells were metabolically labeled with low levels of [^{35}P]orthophosphate for 10 or 18 h, respectively, at which time incorporation of ^{32}P into the β subunit of spectrin had reached steady state (data not shown). The results from three representative experiments with CHO and HeLa JW36 cells are shown in Table III. In CHO cells, there is approximately 1 mol of associated phosphate per mole of spectrin dimer isolated from attached or trypsin-EDTA-detached interphase cells, compared to ~1.6 mol of phosphate per mole of spectrin dimer isolated from mitotic cells (Table III, experiments 1 and 2). This is an increase of ~40% in the absolute level of phosphate associated with the spectrin β subunit in mitosis, calculated from the data in experiment 2 (Table III). This increase in phosphorylation probably occurs in prophase or early metaphase, since the level of phosphorylation of spectrin isolated from G2-enriched popula-
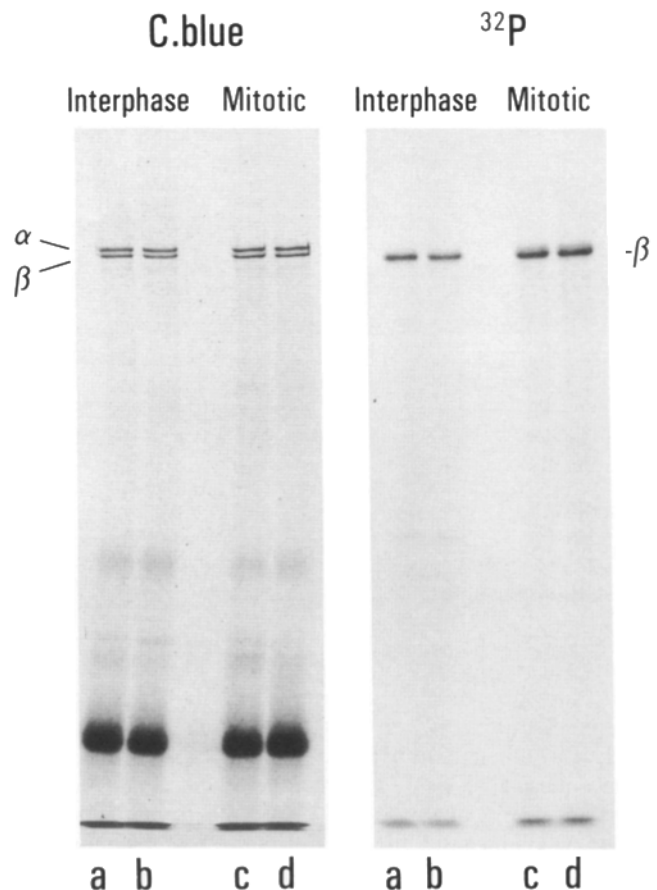

Figure 6. Immunoprecipitation of ^{32}P-labeled spectrin from interphase (lanes a and b) and mitotic (lanes c and d) CHO cells. (Left) Coomassie blue (C. blue)-stained gel. (Right) Corresponding autoradiogram of ^{32}P-labeled spectrin (^{32}P). Lanes a and b, and c and d are duplicate samples of spectrin immunoprecipitates from interphase and mitotic cell extracts, respectively. CHO cells were grown attached to plastic in medium containing 50 μCi/ml [^{32}P]orthophosphate for 5–7 h and spectrin was immunoprecipitated from extracts of asynchronous interphase cells harvested by trypsin-EDTA or from extracts of mitotic cells harvested by mechanical shake-off from attached cultures (10^7 cells/sample). After electrophoresis of the immunoprecipitated spectrin on an SDS gel and staining with Coomassie blue (left), the gel was dried and exposed to x-ray film with an intensifying screen at –70°C to detect ^{32}P (right).

tions of cells is similar to that of the average interphase state (Table III, experiment 2). In HeLa JW36 cells, there is ~1.4 mol of phosphate per mole of spectrin dimer in attached or trypsin-EDTA-detached interphase cells, compared to ~2.4 mol of phosphate per mole of spectrin dimer in mitotic cells (Table III, experiment 3). This represents an increase of ~70% in the level of phosphorylation of the spectrin β subunit.

We have used two-dimensional tryptic peptide mapping to compare the sites of phosphorylation on the spectrin β subunit in interphase and mitotic cells. One, or possibly two, poorly resolved phosphorylated tryptic peptides are observed in the map of spectrin isolated from asynchronous, trypsin-EDTA-detached interphase CHO cells (Fig. 8, top). In contrast, at least three new phosphorylated peptides are clearly evident in the map of spectrin isolated from mitotic
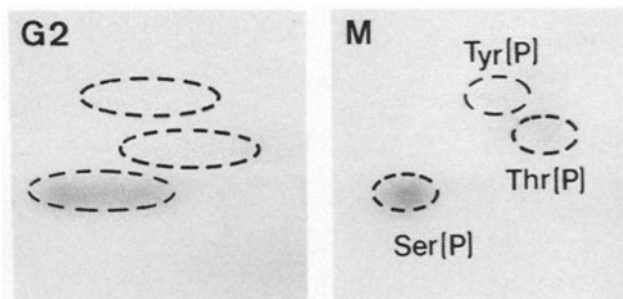
Figure 7. Phosphorylated amino acids present in the spectrin β polypeptide from G2-enriched (*G2*) or mitotic (*M*) CHO cells. Spectrin was immunoprecipitated from extracts of ^{32}P-labeled, G2-enriched, or mitotic CHO cells and subjected to two-dimensional phosphoamino acid mapping as described in Materials and Methods. G2-enriched cells were harvested by trypsin-EDTA and mitotic cells were harvested by mechanical shake-off from attached cultures. Approximately equal ^{32}P counts were analyzed for each sample. The origin is at the lower right with electrophoresis towards the left and chromatography towards the top of each plate. The migration positions of phosphoserine, phosphothreonine, and phosphotyrosine are indicated by the broken circles on the maps. The elongated appearance of the spots on the G2 map is a technical artifact due to smearing at the origin before electrophoresis.

CHO cells (Fig. 8, *bottom*, *arrowheads*). To determine whether the major unmarked spot in the mitotic map migrates in the same position as the spot(s) on the interphase map, mixing experiments will be necessary. For these experiments, interphase and mitotic cells were labeled with ^{32}P for only 1 h. Thus, the peptide maps describe the rate of phosphorylation of specific tryptic peptides, but not necessarily the absolute level of phosphate associated with each peptide. Also, each phosphorylated peptide may contain more than one phosphorylation site.

Together, these experiments indicate that spectrin undergoes a site-specific increase in phosphorylation on the β subunit in mitosis. This appears to be independent of rounding and detachment of the cells from the substratum since (*a*) the molar level of spectrin phosphorylation is the same in attached cells and in cells detached by trypsin-EDTA; and (*b*) the phosphopeptide map of spectrin isolated from mitotic cells is different from the phosphopeptide map of spectrin isolated from trypsin-EDTA–detached cells. In future experiments, it will be important to map spectrin phosphorylation sites in interphase and mitotic cells grown in spinner culture.

Discussion

Spectrin Phosphorylation and Redistribution in Mitosis

We report here that spectrin redistributes from a predominantly insoluble membrane and cytoskeleton-associated fraction in interphase CHO and HeLa cells to a predominantly soluble cytosolic fraction in mitotic cells. Redistribution of spectrin is correlated with mitosis-specific phosphorylation of β spectrin at several new sites and a modest increase in the molar level of β spectrin phosphorylation (a 40 or 70% increase in CHO or HeLa JW36 cells, respectively). We pro-
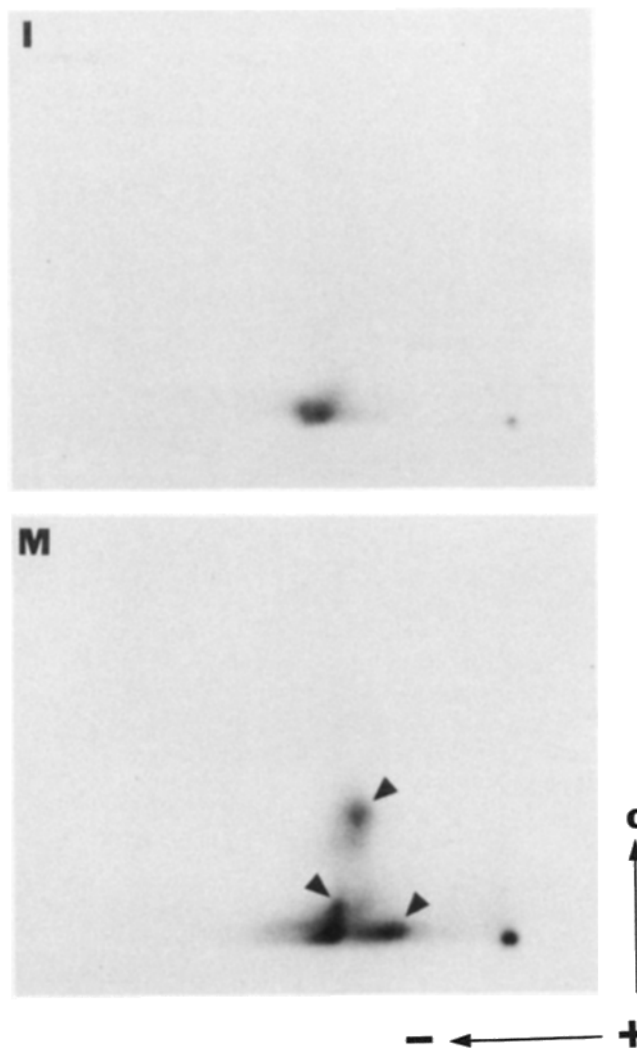

Figure 8. Two-dimensional tryptic peptide maps of ^{32}P-labeled spectrin β chain from interphase (*I*) and mitotic (*M*) CHO cells. Spectrin was immunoprecipitated from extracts of asynchronous ^{32}P-labeled interphase (*I*) CHO cells harvested by trypsin-EDTA or from mitotic (*M*) CHO cells isolated by mechanical shake-off from attached cultures, and then subjected to trypsin digestion and peptide mapping as described in Materials and Methods. Since the amount of spectrin immunoprecipitated from the ^{32}P-labeled mitotic cells was much less than that immunoprecipitated from the ^{32}P-labeled interphase cells (due to a low yield of ^{32}P-labeled mitotic cells in this experiment), unlabeled bovine brain spectrin was included as a carrier in the mitotic gel sample to approximately equalize the amount of spectrin to that in the interphase sample. The interphase maps were exposed for 16 h and the mitotic maps for 10 d. The origin is at the lower right of each plate. Arrowheads on the mitotic map indicate new spots as compared with the interphase map. We were unable to improve the separation of these peptides by altering the times of electrophoresis and chromatography, or by using chymotrypsin to generate the peptide maps.

pose that phosphorylation of β spectrin in mitosis could cause spectrin to dissociate from ankyrin or other membrane-binding site(s) as well as from actin filaments, thus leading to disassembly of the spectrin-based membrane skeleton. This idea is suggested by our observations that the amount of soluble spectrin in mitotic cells does not depend on the presence of detergent (Triton X-100) and that soluble

spectrin in mitotic extracts cosediments with purified brain spectrin tetramers on sucrose gradients.

Phosphorylation of erythrocyte β spectrin by cAMP-independent protein kinase has been reported to have no effect on spectrin-actin interactions or spectrin dimer-tetramer equilibrium (Brenner and Korn, 1979; Ungewickell and Gratzer, 1978; Mische, S. M., and J. S. Morrow, unpublished results), although more recent studies suggest that phosphorylation may stabilize the association of erythrocyte spectrin to higher order oligomers (Mische, S. M., and J. S. Morrow, unpublished results). However, these studies of erythrocyte spectrin phosphorylation may not be an appropriate model for nonerythroid spectrin interactions in mitosis since (a) the effect of phosphorylation by mitotic kinases on erythrocyte spectrin interactions has not been investigated; (b) the predominant β-spectrin polypeptide expressed in most nonerythroid cell types (β-fodrin) is the product of a different gene (Bennett, 1990a,b; Morrow, 1989); and (c) in tissues in which the erythroid β-spectrin gene is expressed, the extreme COOH-terminal end of erythrocyte β-spectrin that contains the phosphorylation sites (Harris and Lux, 1980) is spliced out and is replaced by a different COOH-terminal sequence, which is missing at least one of the previously identified phosphorylation sites (Winkelmann et al., 1990).

Direct experiments to test the functional consequences of mitotic phosphorylation on nonerythroid spectrin binding to ankyrin, membranes, and actin filaments will be feasible once the identity of β-spectrin mitotic kinase(s) has been established. β-Spectrin in mitotic cells could be phosphorylated directly by the mitotic kinase, p34^{cdc2}, as are nuclear lamins, vimentin intermediate filaments, and the actin-binding protein caldesmon (for reviews see Lewin, 1990; Pines and Hunter, 1990; Sobue and Sellers, 1991). Tyrosine kinases such as p60^{src} can be excluded since β-spectrin phosphorylation is exclusively on serine residues. In addition to comparison of phosphopeptide maps of β-spectrin isolated from mitotic cells with maps of β-spectrin phosphorylated in vitro by p34^{cdc2} (or other kinases), it will be important to analyze relevant β-spectrin (β-fodrin) sequences (once they are available) for the presence of the consensus phosphorylation motif characteristic of the p34^{cdc2} protein kinase (Pines and Hunter, 1990).

Spectrin Redistribution and Formation of Microvilli in Mitotic Cells

Immunofluorescence localization of spectrin in mitotic CHO cells reveals that spectrin is predominantly localized in the cytoplasm. Spectrin is not associated with the plasma membrane in the numerous actin filament-containing microvilli that cover the cell surface, nor is it found in the longer actin-containing filopodia that link the round mitotic cells to the culture dish (see Figs. 3 and 4). In contrast, in well-spread, flat interphase cells attached to plastic, spectrin staining appears to be predominantly associated with the dorsal and ventral plasma membranes and is enriched at cell-cell contact sites (Fig. 2). Together with the biochemical data demonstrating an increase in soluble spectrin in mitosis, these observations are consistent with the possibility that spectrin dissociates from the plasma membrane when cells enter mitosis (although dissociation of some spectrin from intracellular membranes or cytoskeletal components is not

ruled out). This could lead to partial disassembly of spectrin-associated cortical actin filament networks, facilitating the recruitment of actin filaments into microvilli that exclude spectrin and contain other actin-binding proteins. Exclusion of spectrin from actin filaments in microvillar membrane domains is also characteristic of the microvilli that appear transiently on the surface of A-431 cells after stimulation by EGF (Bretscher, 1989), as well as the stable microvilli in the brush border of intestinal epithelial cells (Glenney et al., 1983; Hirokawa et al., 1983) and the stereocilia (microvilli) in the hair cells in the inner ear (Holley and Ashmore, 1990; Drenckhahn et al., 1991). Under some conditions, spectrin has been reported to be associated with the actin filaments in microvilli in isolated cortices from activated sea urchin eggs (Fishkind et al., 1990a). This may be due to differences in microvillar structure between sea urchin eggs and other eukaryotic cells.

Formation of microvilli in mitotic cells has been proposed to provide a mechanism to store excess membrane surface area that is generated when large flat interphase cells round up before cleavage (Erickson and Trinkaus, 1976; Porter et al., 1973, 1974; Sanger et al., 1984), and to provide the extra surface area needed for a suspension-grown, spherical cell to divide in two (Graham et al., 1973; Knutton et al., 1975). However, cells in which spectrin aggregation was induced by microinjection of antispectrin antibodies rounded up and proceeded normally through mitosis and cell division (Mangeat and Burridge, 1984). This suggests that spectrin redistribution and dissociation from the plasma membrane may not be required for cell rounding, microvilli formation, or cleavage but instead might be a consequence of cell rounding and microvilli formation in mitotic cells. It would be interesting to determine whether preventing spectrin dissociation from the membrane would have any effects on these mitotic events. Alternatively, disassembly of spectrin-associated cortical actin filament networks could be important for the cessation of endocytosis and exocytosis characteristic of mitotic cells (Warren, 1985), or for changes in the mechanical properties of mitotic cells (Salmon, 1989).

Spectrin Redistribution and Reduction of Cell Contacts in Mitotic Cells

For mitotic cells harvested by mechanical shake-off from cells growing in monolayer culture, the increase in Triton-soluble spectrin may be partly a consequence of reduction in cell-cell and/or cell-matrix contacts and partly a consequence of mitosis-specific events. This is suggested because the amount of soluble spectrin in interphase CHO or HeLa cells detached from the substratum by trypsin-EDTA or by growing in suspension (25-38%) is intermediate between mitotic cells (60%) and interphase cells growing attached in monolayer culture (11-15%) (see Table II). However, unlike entry into mitosis, redistribution of spectrin induced by cell detachment is not correlated with a change in the level of phosphorylation of β-spectrin.

It is tempting to speculate that spectrin in CHO and HeLa cells could be associated with two different membrane-binding sites, with one linkage sensitive to cell-cell (or cell-matrix) interactions and the other linkage sensitive to cell contact-independent events specific for mitosis. Spectrin interactions with ankyrin or ankyrin-independent membrane-binding sites could be differentially regulated by phos-

phorylation by mitotic kinases or by alternative signaling pathways triggered by changes in cell adhesion status. For example, interactions of spectrin with ankyrin-independent membrane-binding sites (but not with ankyrin) are downregulated by Ca^{2+}/calmodulin (Steiner et al., 1989). Candidate membrane proteins to which spectrin might be linked via ankyrin include the $Na^+/K^+ATPase$, anion channels, or cadherins; candidate membrane proteins to which spectrin might be linked by ankyrin-independent interactions are not well characterized (for reviews see Bennett, 1990a,b; Rodriguez-Boulan and Nelson, 1989; Morrow, 1989). A clearer understanding of the mechanisms and functional significance underlying spectrin redistribution in mitosis in CHO and HeLa cells will be achieved once the membrane-binding site(s) for spectrin have been identified in these cell types.

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