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Membrane Association of a 36,000-Dalton Substrate for Tyrosine Phosphorylation in Chicken Embryo Fibroblasts Transformed by Avian Sarcoma Viruses

KATHRYN RADKE, V. CELESTE CARTER, PAUL MOSS, PHILIP DEHAZYA, MANFRED SCHLIWA, and G. STEVEN MARTIN
Department of Zoology, University of California, Berkeley, California 94720

ABSTRACT A cellular protein of 36,000 daltons becomes phosphorylated at tyrosine in chicken embryo fibroblasts transformed with avian sarcoma viruses. We have used cellular fractionation and immunofluorescence to locate the 36-kdalton protein in virus-transformed and uninfected chicken fibroblasts. The 36-kdalton protein in transformed cells fractionated mainly with high-speed particulate material, and in density gradient separations, the 36-kdalton protein was found in association with light density membranes together with most of the plasma membrane marker. Increasing the concentration of salt or adding ion chelators solubilized some of the 36-kdalton protein that otherwise was pelletable with high g forces. Based on these data, we conclude that this protein is peripherally or indirectly attached to light density membranes, including plasma membranes.

Indirect immunofluorescent staining of the 36-kdalton protein in fixed cells revealed that it was located inside the cell in an extensive reticulum apposed to surface membranes. The same pattern of staining was found in both uninfected and virus-transformed cells. Pretreatment of cells with nonionic detergents before fixation altered or abolished 36-kdalton staining. The 36-kdalton protein appeared to be excluded from regions of the cells where actin cables were present. The pattern of staining observed with the anti-36-kdalton antibody was similar, but not identical, to that observed with antiserum against nonerythroid spectrin. Thus, the data obtained by biochemical fractionation and by immunofluorescent staining indicate that the 36-kdalton protein is found in a reticulum at the inner surface of the plasma membrane, possibly in association with cytoskeletal proteins.

Oncogenic transformation of cells by a number of avian sarcoma viruses is mediated by viral proteins with associated tyrosine-specific protein kinase activities (21). Cells transformed by Rous, Fujinami, PRC II and Y73 sarcoma viruses exhibit 3–10-fold increases in the phosphotyrosine content of cellular proteins (3, 33, 41). Transformation of cells by these viruses may thus result from the altered phosphorylation of cellular proteins whose normal functions involve the maintenance of structure and the control of growth.

Several cellular substrates of the viral tyrosine protein kinases have been identified (11, 20, 35, 42). An abundant cellular protein of 36,000 daltons is a major substrate for tyrosine phosphorylation in Rous sarcoma virus (RSV)[1]-transformed fibroblasts (16, 37), myotubes (25), and neuroretinal cells (34). The 36-kdalton protein is also phosphorylated at tyrosine following transformation of cells by other avian sarcoma viruses (12, 15, 33). The function of the 36-kdalton protein and the role of its phosphorylation in transformation are unknown. Examination of the properties of cells infected by mutants of RSV that differ in their effects on various parameters of transformation has indicated that tumorigenicity, increased production of plasminogen activator protease,

[1] *Abbreviations used in this paper:* CEF, chicken embryo fibroblasts; D, -dimensional; FITC, fluorescein isothiocyanate; FSV, Fujinami sarcoma virus; LDH, lactate dehydrogenase; NADH, reduced nicotinamide-adenine dinucleotide; P150, particulate fraction; PHEM, 0.06 M PIPES, 0.025 M HEPES, 0.01 M EGTA, and 0.002 M $MgCl_2$, pH 6.9; RSV, Rous sarcoma virus; S150, soluble fraction; TK, 0.02 M Tris-HCl pH 7.2, 0.005 M KCl, and 1% vol/vol aprotinin; TKM, TK buffer plus 0.001 M $MgCl_2$.

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and loss of anchorage dependence for growth are correlated with phosphorylation of the 36-kdalton protein (23, 32).

A knowledge of the location of the 36-kdalton protein may provide clues about its function. We previously showed that the 36-kdalton protein is present in the cytoplasts of enucleated cells (36). Following extraction of cells with nonionic detergent in a hypertonic sucrose buffer reported to stabilize structural elements of the cell, the 36-kdalton protein remained in the detergent-insoluble matrix (10, 13). In addition, the 36-kdalton protein was present in the particulate fraction of cell extracts prepared in hypotonic buffers (13). We have used an antiserum to locate the 36-kdalton protein by cellular fractionation and immunofluorescence in both virus-transformed and uninfected chicken embryo fibroblasts (CEF). The results of cellular fractionation experiments indicate that the 36-kdalton protein is peripherally or indirectly attached to light density membranes that include plasma membranes. Staining the 36-kdalton protein by indirect immunofluorescence revealed that the protein is located inside the cell in an extensive reticulum apposed to surface membranes. In uninfected fibroblasts, the 36-kdalton protein is excluded from areas occupied by actin microfilament bundles. Detergent extraction before fixation grossly alters or abolishes the 36-kdalton staining, but does not alter the distribution of actin microfilament bundles. No alteration in the location of the 36-kdalton protein in virus-transformed cells is detected by the technique of indirect immunofluorescence.

MATERIALS AND METHODS

Virus Infection and Cell Growth: CEF were infected with the Schmidt-Ruppin strain of Rous sarcoma virus (RSV), subgroup A or D, with a wild-type, temperature-resistant strain of Fujinami sarcoma virus (*tr*FSV; reference 30), or with a temperature-sensitive strain of FSV (*ts*FSV; reference 33). Infected cells were cultured in Dulbecco's modified Eagle's medium (DME) supplemented with 10% tryptose phosphate broth, 4% calf serum, and 1% chicken serum, for 2–3 d at 38°C, then subcultured at 2×10^6 cells per 100-mm plate and labeled 2 d later. Uninfected cells were subcultured at 1×10^5 cells per 16-mm well or 5×10^5 cells per 35-mm well and labeled the next day.

Radiolabeling of Cellular Proteins: Exponentially growing cells were radiolabeled with [^{35}S]methionine for 6 h in methionine-free DME supplemented with 4% calf serum and 1% chicken serum or for 18 h in the same medium prepared with 5–15% complete DME. The temperature during labeling was 38°C unless noted otherwise. Cells in 16- or 35-mm wells were labeled in 0.25 or 1.0 ml containing 50–200 μCi [^{35}S]methionine/ml, and cells in 100-mm plates were labeled in 5 ml with 50 μCi [^{35}S]methionine/ml medium.

Preparation of an Antiserum Against the 36-kdalton Protein: The 36-kdalton protein was purified from uninfected CEF essentially as described by Erikson and Erikson (16). The purity of the preparation was confirmed by two-dimensional (2D) polyacrylamide gel electrophoresis (35). Analysis of the purified protein on a 2D gel revealed that the 36-kdalton protein was the only Coomassie Blue–stained species found between M_r 10,000 and 250,000, and within the wide range of isoelectric points examined by nonequilibrium pH gradient electrophoresis (35). The 36-kdalton protein resolved as two spots of the same molecular weight. An antiserum was prepared in an adult New Zealand white rabbit by subcutaneous injection of 100 μg of purified 36-kdalton emulsified in complete Freund's adjuvant. The animal was boosted at 3 and 7 wk using the same procedure. Sera obtained either 10 d or 4 wk after the first boost were used in the experiments reported here.

Immunoprecipitation: Either of two detergent buffers was used to solubilize proteins from intact cells or from mechanically disrupted cells. Nonidet P40 lysis buffer contained 0.1 M NaCl, 0.01 M Tris-HCl pH 7.4, 0.001 M EDTA, 1 mg/ml bovine serum albumin, 1% wt/vol NP40 and 1% vol/vol aprotinin (Sigma Chemical Co., St. Louis, MO). RIPA lysis buffer contained 0.15 M NaCl, 0.01 M Tris-HCl pH 7.4, 0.001 M EDTA, 1% wt/vol Nonidet P40, 1% wt/vol Na deoxycholate, 0.1% wt/vol SDS and 1% vol/vol aprotinin. Both buffers extracted the 36-kdalton protein equally well.

Chilled cell cultures were washed with cold PBS, then extracted with 1 ml of lysis buffer per 10^6 cells. After 10 min at 0°C, the lysates were clarified by centrifugation at 13,000 *g* for 10 min at 4°C. Portions of cellular homogenates or subcellular fractions were diluted with an equal volume of double strength lysis buffer, then diluted with single strength lysis buffer so that the material from 10^6 cells was suspended in 0.5 ml. The samples were stored at −70°C and clarified before use.

Portions of fractions containing the material from 10^5 cells were incubated with 2 μl of rabbit anti–36-kdalton serum for 30 min at 0°C. (1 μl of serum was sufficient to precipitate all the 36-kdalton protein from 10^5 cells under these conditions.) Antigen-antibody complexes were adsorbed for 15 min at 0°C with 20 vol of a 10% suspension of fixed, protein A-containing *Staphylococcus aureus* per volume of antiserum. The fixed bacteria had first been washed in lysis buffer and resuspended in a detergent lysate of unlabeled CEF to eliminate nonspecific binding of radioactive proteins. The bacteria and adsorbed immune complexes were washed sequentially with high salt, high detergent, and low salt buffers as previously described (17). Immune complexes were solubilized in SDS gel sample buffer or in isoelectric focusing sample buffer (35).

Immunoprecipitated proteins were separated as described (35) by electrophoresis on 10% SDS polyacrylamide gels or by 2D gel electrophoresis using nonequilibrium pH gradient electrophoresis (pH 3.5–10 or pH 6–9.5) in the first dimension. When necessary, gels were impregnated with Enhance (New England Nuclear, Boston, MA) before exposure to Kodak XAR film. Bands containing the 36-kdalton protein were excised from gels and digested at 60°C for 2 h in 0.7 ml NCS solubilizer (Amersham Corp., Arlington Heights, IL). The mixture was diluted with 10 ml Omnifluor (New England Nuclear) and, after 24 h, the radioactivity was quantitated by liquid scintillation counting.

Characterization of the Antiserum Against the 36-kdalton Protein: The specificity of the rabbit anti–36-kdalton serum was examined by immunoprecipitating [^{35}S]methionine-labeled proteins from detergent lysates of CEF. The precipitates, analyzed on SDS-polyacrylamide gels, contained one major polypeptide band of M_r 36,000 (Fig. 1 *A*). This band was not present in precipitates of the preimmune rabbit serum. Several bands of other molecular weights and of relatively minor abundance were precipitated by both sera, probably as a result of nonspecific binding to the bacterial adsorbent. This inference is supported by the finding that only one band of 36,000 M_r is stained by the anti–36-kdalton serum in immunoblots of whole cell lysates (data not shown). The anti–36-kdalton serum also immunoprecipitated ^{32}P-labeled 36-kdalton phosphoprotein from lysates of RSV-transformed cells.

To further confirm the specificity of the rabbit anti–36-kdalton serum, we used 2D gels to examine immunoprecipitates of [^{35}S]methionine-labeled CEF polypeptides. The precipitates contained a major spot of M_r 36,000 (Fig. 1 *B*) that co-migrated with the 36-kdalton polypeptide of uninfected CEF when mixed with whole-cell lysates (data not shown). Two species of 36-kdalton phosphoprotein (containing one and two phosphates per polypeptide) that we previously identified in whole-cell lysates (37) were present in immunoprecipitates of proteins from RSV-transformed cells (Fig. 1 *D*). Quantitative comparison on 2D gels of immunoprecipitated 36-kdalton polypeptides with those from whole-cell lysates showed that 20% of the 36-kdalton protein was phosphorylated in RSV-transformed CEF analyzed in either manner. Analysis of immunoprecipitates on 1D SDS gels showed that the antiserum precipitated as 36-kdaltons ~0.35% of the total methionine-labeled polypeptides, as expected from analysis of 2D gels of whole cell polypeptides that were run in parallel. Thus, in antibody excess, the 36-kdalton protein is quantitatively immunoprecipitated from cell extracts.

In addition to the major species of 36-kdalton protein previously identified in whole cell lysates of uninfected or RSV-transformed cells, minor species were also detected in the immunoprecipitates (Fig. 1, *B* and *D*). These appear to be derived from the major nonphosphorylated or phosphorylated forms by the addition of a single negative charge. Since no polypeptides of similar relative abundance were apparent in whole-cell lysates (Fig. 1 *C*), we presume that these minor species are charge derivatives generated during cellular lysis in the absence of the high concentrations of urea and mercaptoethanol used to prepare samples for 2D gels. All were shown by V8 mapping to be authentic 36-kdalton polypeptides. No unrelated polypeptides of M_r 36,000 were precipitated by the anti–36-kdalton serum (Fig. 1 *B*). Thus we conclude that the rabbit anti–36-kdalton serum used in this work specifically immunoprecipitates only the 36-kdalton protein from extracts of CEF.

Preparation of Homogenates: Homogenates were prepared as described by Courtneidge et al. (14). All procedures were performed at 0–4°C. 5–10 subconfluent 100-mm plates, each containing ~6×10^6 cells, were washed with cold PBS and the cells were removed from the substrate by gentle scraping with a soft rubber policeman. After pelleting at 1,000 *g*, cells were swollen for 15 min in hypotonic buffer and broken open with a tight-fitting Dounce homogenizer at concentrations of $1–2 \times 10^7$ cells/ml. Hypotonic buffers contained 0.02 M Tris-HCl pH 7.2, 0.005 M KCl, and 1% vol/vol aprotinin (TK buffer) or TK buffer plus 0.001 M $MgCl_2$ (TKM buffer). Isotonic buffers contained 0.15 M NaCl in addition. 15–20 strokes of the pestle were sufficient to break open 90–95% of the cells in hypotonic buffer without damaging nuclei. In isotonic buffer, 40–50 strokes were required and some nuclei were damaged.

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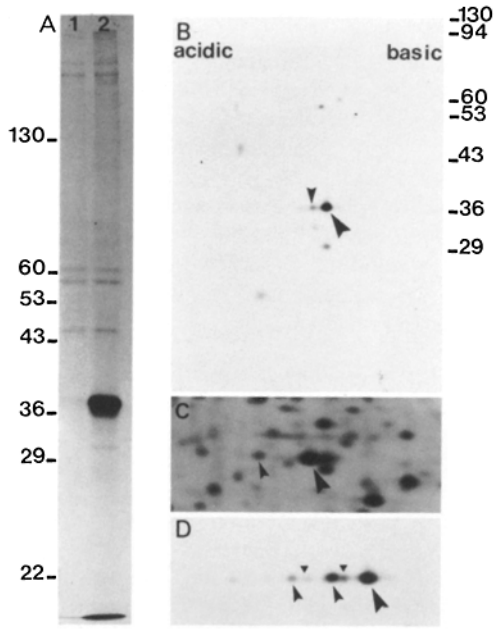

FIGURE 1 Polyacrylamide gel analysis of immunoprecipitated 36-kdalton protein. (*A*) Proteins from [^{35}S]methionine-labeled uninfected CEF were extracted with NP40 lysis buffer and immunoprecipitated with equal amounts of preimmune or anti-36-kdalton serum. Immunoprecipitates were resolved on a 10% SDS polyacrylamide gel and the radioactive polypeptides detected by autoradiography. Lane *1*, preimmune serum; lane *2*, anti-36-kdalton serum. Molecular weights ($\times 10^{-3}$) of Coomassie Blue-stained standards are indicated at the left. (*B*) A precipitate similarly obtained with anti-36-kdalton serum was resolved by 2D gel electrophoresis with nonequilibrium pH gradient electrophoresis (pH 3.5–10) in the first dimension. A portion of an autoradiogram of the second dimension, 10% SDS gel is shown with the acidic end of the 1D gel on the left. The species of 36-kdalton protein that co-migrates with the 36-kdalton protein on 2D gels of whole-cell lysates is marked with a large diagonal arrowhead. The acidic charge derivative described in the text is marked with a small arrowhead. (*C*) Polypeptides of a whole-cell lysate of [^{35}S]methionine-labeled, RSV-transformed CEF were resolved on 2D gels using nonequilibrium pH gradient electrophoresis (pH 6–9.5) in the first dimension. A small portion of the autoradiogram containing the nonphosphorylated (large diagonal arrowhead) and phosphorylated (small diagonal arrowhead) 36-kdalton polypeptides is shown. (*D*) [^{35}S]Methionine-labeled polypeptides from RSV-transformed CEF were immunoprecipitated with anti-36-kdalton serum and resolved on 2D gels as for *B*. The portion of the autoradiogram shown contains the nonphosphorylated 36-kdalton (large diagonal arrowhead), two forms of the 36-kdalton phosphoprotein (small diagonal arrowheads), and their single-charge derivatives (small vertical arrowheads).

Breakage was monitored by phase microscopy.

Fractionation of Homogenates: Homogenates were separated into particulate (P150) and soluble (S150) fractions by high speed centrifugation. In most cases, nuclei and remaining whole cells were first removed by centrifugation at 1,500 rpm in a Sorvall HB4 rotor (375 *g*; DuPont Instruments-Sorvall Biomedical Div., DuPont Co., Wilmington, DE) for 10 min at 2°C. The nuclear pellet was dispersed once or twice more either by rehomogenization and aspiration through a 25- or 26-gauge needle, or simply by aspiration through the needle. The washed nuclear pellet was resuspended in homogenization buffer and was analyzed for marker enzymes, for protein content, and for the 36-kdalton protein. To obtain P150 and S150 fractions, the pooled postnuclear supernatants were centrifuged in thick-walled polyallomer tubes at 40,000 rpm in a Beckman SW50.1 rotor (150,000 *g*) for 1 h at 0°C. For direct analysis, the P150 pellet was resuspended in homogenization buffer with a 26-gauge needle.

Fractionation of the material in the P150 on discontinuous sucrose gradients was performed as described by Courtneidge et al. (14) with the following modifications. Material in the pellet was resuspended in 2.5 ml gradient buffer (0.02 M Tris-HCl pH 7.2, 0.005 M KCl, 0.005 M NaCl, 0.1 mM EDTA and 1% vol/vol aprotinin) by aspirating it five times through a 26-gauge needle and then by five strokes in a Dounce homogenizer. The sample was loaded on top of a gradient containing 2.2-ml layers of 20%, 35%, 40%, and 50% wt/vol sucrose in gradient buffer. 0.1 mM EDTA was included in the sucrose solutions to prevent aggregation of protein in the gradients; at this concentration, EDTA does not dissociate ribosomes from the rough endoplasmic reticulum (14). After centrifugation at 200,000 *g* for 2 h at −2°C (40,000 rpm in a Beckman SW41 rotor; Beckman Instruments, Inc., Spinco Div., Palo Alto, CA), the material at the sucrose-sucrose interfaces was collected, diluted to 11.5 ml in homogenization buffer, and pelleted at 40,000 rpm in the SW41 rotor for 45 min at 0°C. The resulting pellets were resuspended in homogenization buffer by aspiration through a 26-gauge needle for further analysis.

Fractionation of postnuclear supernatants on discontinuous sucrose gradients was performed as described for the P150 pellets, except that the supernatants were loaded in homogenization (TK or TKM) buffers or in homogenization buffers supplemented with 0.15 M NaCl. After centrifugation of the gradients, a volume equivalent to the original supernatant was removed from the top of the gradient and saved for analysis. Material that migrated to sucrose-sucrose interfaces did not form tight bands like those obtained by separation of P150 pellets. Therefore, the entire sucrose layer above a given interface was harvested together with the material at the interface. Samples from the gradient were diluted in the same buffer that had been used to prepare the postnuclear supernatant and pelleted in the SW41 rotor at 40,000 rpm for 110 min at 0°C. The resulting pellets were resuspended in the same buffer for analysis.

Marker Enzyme Assays: Plasma membrane content was determined by measuring 5′-nucleotidase activity (29). Deoxycholate or the zwitterionic detergent, sulfobetaine-14 (Calbiochem-Behring Corp., La Jolla, CA), was added to samples at 0.2% or 0.5% wt/vol, respectively, to solubilize 5′-nucleotidase (2). Samples were taken up in TKM buffer and incubated for 30 min at 37°C in assay buffer (100 mM glycine-NaOH, pH 9.0, 10 mM $MgCl_2$, 0.1 mM AMP) containing [^{3}H]AMP at a concentration of 2 μCi/ml. Unhydrolyzed AMP was precipitated with $Ba(OH)_2$ and $ZnSO_4$ (26) and the amount of free [^{3}H]adenosine in the supernatant was determined by liquid scintillation counting.

Lactate dehydrogenase (LDH) activity (24) was used to monitor soluble protein after extracts were frozen and thawed to break open any remaining whole cells. Reduced nicotinamide-adenine dinucleotide (NADH) diaphorase activity (1) was used to monitor endoplasmic reticulum. Protein was measured by the Bradford assay (7).

Immunofluorescence: Uninfected or RSV-transformed CEF (1×10^4 cells) were seeded onto 12-mm glass coverslips and used 18 h later for immunofluorescent staining. The cells were washed once with PHEM buffer (0.06 M PIPES, 0.025 M HEPES, 0.01 M EGTA and 0.002 M $MgCl_2$, pH 6.9; reference 39) and fixed with 2% paraformaldehyde in PHEM buffer for 20 min at room temperature. Fixed cells were then permeabilized either with 0.1% (vol/vol) Triton X-100 in PHEM buffer for 20 min at room temperature or with absolute acetone for 7 min at −20°C. In some experiments, cells were extracted before fixation either with 0.1% (vol/vol) Triton X-100 in PHEM buffer for 1 min or with 0.2% (wt/vol) Brij-58 in PHEM buffer for 5 min at room temperature (40). Fixed and permeabilized cells were washed once in PHEM buffer and three times in PBS. Each coverslip was incubated with 12 μl of a 1:80 dilution of rabbit anti-36-kdalton serum or pre-immune serum in a moist chamber for 45 min at room temperature. After three washes in PBS, the coverslips were incubated with 12 μl (25 μg/ml) of fluorescein (FITC)-conjugated goat anti-rabbit immunoglobulin G (Sigma Chemical Co.) for 45 min at room temperature. A rabbit antiserum against pig brain spectrin, kindly provided by K. Burridge (reference 9; Univ. North Carolina, Chapel Hill, NC), was used at a 1:20 dilution on CEF and at a 1:100 dilution on mammalian cells with the same procedures as for the anti-36-kdalton serum. In actin/36-kdalton protein double fluorescence experiments, the coverslips were washed extensively with PBS and incubated for 45 min at room temperature with rhodamine-conjugated phalloidin (20 μg/ml) that was kindly provided by T. Wieland (reference 47; Max Planck Institute for Medicine, Heidelberg, Federal Republic of Germany). Stained preparations were mounted in PBS-glycerol containing the anti-bleaching agent *p*-phenylenediamine (22) and viewed with a Zeiss photomicroscope III using Planchromat 40×/0.65 and Planapochromat 63×/1.4(oil) objectives with appropriate barrier filters. Photographs were made with Kodak Plus-X film that was developed with Ilford Microphen developer.

RESULTS

Distribution of the 36-kdalton Protein among Subcellular Fractions of Transformed Cells

Virus-transformed CEF that had been swollen in hypotonic TKM buffer were broken open with a Dounce homogenizer

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and the homogenates were cleared of nuclei and remaining intact cells by low speed centrifugation. The postnuclear supernatants were then separated by centrifugation into high speed P150 and soluble S150 fractions. The amount of 36-kdalton protein in each fraction was quantitated by immunoprecipitating [^{35}S]methionine-labeled protein with excess antibody and then counting the isotope present in 36-kdalton bands cut from SDS gels of the immunoprecipitates.

The 36-kdalton protein was mainly associated with the postnuclear particulate fraction and the nuclear pellet; a variable amount (1–30%) was soluble (Table I). The distribution of the 36-kdalton protein was similar to that of the plasma membrane marker, 5′-nucleotidase, and was clearly different from that of total protein, the soluble marker, LDH, and the endoplasmic reticulum marker, NADH diaphorase. In many experiments, more 36-kdalton protein was present in the nuclear pellet than could be accounted for by the presence of whole cells, as judged by LDH activity, or by the presence of plasma membranes. This may reflect an association of the 36-kdalton protein with nuclei in a structure that was not reliably preserved by this type of fractionation. The distribution of the 36-kdalton protein was the same in fusiform cells transformed by *tr*FSV and in round cells transformed by RSV(SR-D) or by *ts*FSV and RSV together. Similarly, there was no effect of growth temperatures of 35°C, 38°C, or 41°C, or of durations of radiolabeling of 6 or 18 h on its subcellular distribution.

Particulate fractions prepared in this way contain large fragments of plasma membrane, intracellular membranes such as endoplasmic reticulum and Golgi, and organelles such as lysosomes and mitochondria (14, 27, 29). To investigate the association of the 36-kdalton protein with specific components of the particulate fraction, we separated the material in the P150 on four-step discontinuous sucrose gradients (14). 84% of the 36-kdalton protein was found at the 20%/35% sucrose interface, where the bulk of the plasma membrane marker was also located (Table II). Little 36-kdalton protein was found in the heaviest membrane fraction at the 40%/50% interface, where half the endoplasmic reticulum marker was located (Table II). Mitochondria and Golgi membranes have also been reported to be present at this interface (14). Thus, the 36-kdalton protein present in resuspended P150 fractions specifically and repeatably associated with the light density membrane fraction that contains plasma membranes as well as some smooth endoplasmic reticulum. This distribution of radiolabeled 36-kdalton protein was not influenced by the shape of the transformed cell or by the duration of radiolabeling.

To show that the association of the 36-kdalton protein with light density membranes was not generated by the preparation of a high speed pellet, we loaded postnuclear supernatants prepared from low salt homogenates onto discontinuous sucrose gradients. Nearly all of the 36-kdalton protein cleared from the postnuclear supernatant (Table III). The distribution of the markers within these gradients was somewhat different than that described in Table II. Nevertheless, two-thirds of both the 36-kdalton protein and 5′-nucleotidase activity were found in the light density membrane fraction, and very little of either was found at the 40%/50% sucrose interface. These results confirm the association of the 36-kdalton protein with light density membranes.

Nature of the Association of the 36-kdalton Protein with Light Density Membranes

Proteins associated with, but not integral to, membranes can be removed from fragments of membrane using chelators of divalent cations or by increasing ionic strength. Integral proteins are released with nonionic detergents but not by chelators or salt unless the treatments are so extensive as to cause membrane vesiculation (44). To see whether the 36-kdalton protein behaved as an integral membrane protein, we treated the material in postnuclear supernatants that had been prepared in hypotonic buffer with increasing concentrations of salt. The 36-kdalton protein was increasingly solubilized as the concentration of salt was raised: 3% was soluble in 5 mM KCl with no added NaCl, and 40% was soluble when 800 mM NaCl was added (Fig. 2). Thus, a substantial fraction of the 36-kdalton protein behaved as a peripheral protein. The incomplete solubilization of the 36-kdalton protein by 800 mM NaCl may indicate that higher salt concentrations are required for complete solubilization. However, it raises the possibility that factors in addition to ionic forces may play a role in its association with membranes. Alternatively, some 36-kdalton protein may be dissociated from membranes by high salt in a structure that remains pelletable at 150,000 *g*.

To confirm that some of the 36-kdalton protein behaves as a peripheral protein, material in the P150 pellet was resus-

TABLE II

Distribution of the 36-kdalton Protein Found in the P150 after Centrifugation into Discontinuous Sucrose Gradients*

Sucrose interface	Percentage of total recovered			
	36-kdalton protein	Protein	5′-Nucleotidase	NADH diaphorase
20%/35%	84 (80–88)‡	40	69 (61–93)	27
35%/40%	13 (10–16)	14	19 (5–29)	27
40%/50%	3 (2–4)	46	12 (1–16)	46

* P150 fractions were prepared as described in Table I.
‡ Average and range for three experiments.

TABLE I

*Distribution of the 36-kdalton Protein in Subcellular Fractions of Homogenates Prepared in Hypotonic TKM Buffer**

Fraction	Percentage of total recovered				
	36-kdalton protein	Protein	LDH	5′-Nucleotidase	NADH diaphorase
Nuclear pellet	25 (12–48)‡	22	5	22 (18–25)	10
P150	54 (51–58)	25	6	64 (61–70)	79
S150	21 (1–32)	53	88	16 (12–20)	11

* [^{35}S]Methionine-labeled, virus-transformed cells were homogenized in hypotonic TKM buffer and subcellular fractions were prepared by differential centrifugation as described in the Materials and Methods.
‡ Average and range for three experiments.

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pended in hypotonic buffer with 5 mM EDTA plus 5 mM EGTA, or with 600 mM NaCl. About a quarter more of the 36-kdalton protein was solubilized by these agents than by the mechanical resuspension itself (Table IV). This too suggests that a fraction of the 36-kdalton protein is peripherally or indirectly associated with light density membranes. Nearly all the 36-kdalton protein in the resuspended P150 was solubilized by 1% Triton X-100 (Table IV). This indicates that the 36-kdalton protein is not associated with a detergent-insoluble structure in these pellets.

CONTROLS FOR ARTEFACTUAL ASSOCIATION: It was possible that some of the procedures used might have caused posthomogenization association of the 36-kdalton protein with membranes. Low concentrations of salt are conventionally used to swell cells before breakage in the Dounce homogenizer, allowing the isolation of large fragments of plasma membranes with minimal damage to nuclei (18). However, proteins can artefactually absorb to membranes through weak ionic interactions in such low salt buffers. Similarly, magnesium ions are used to protect nuclei from breakage in hypotonic buffers, but can also cause membrane and protein aggregation (18). We therefore varied the procedures used to prepare cell homogenates. To test the effects of different homogenization buffers, we homogenized virus-transformed CEF in solutions containing 150 mM NaCl, either in the absence or presence of magnesium ions (Table V *a*). In addition, we added salt and/or magnesium ions to homogenates prepared by breaking the cells in low salt buffer without Mg^{2+}

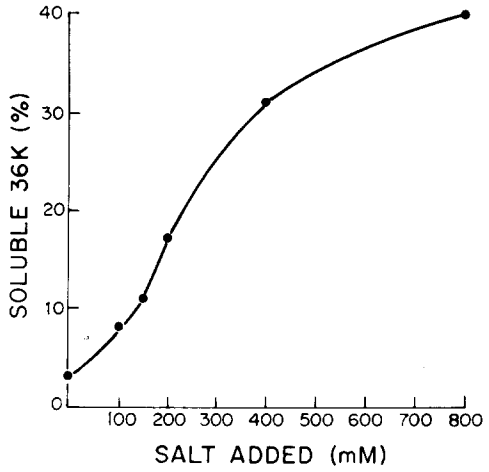

FIGURE 2 Release of the 36-kdalton protein from pelletable material with increasing concentrations of salt. [^{35}S]Methionine-labeled, FSV-transformed CEF were homogenized in hypotonic TK buffer and the nuclei removed by low-speed centrifugation. The postnuclear supernatant was divided into six portions whose volume was adjusted with TK buffer and 3 M NaCl to yield the salt concentrations indicated. The samples were then subjected to centrifugation for 1 h at 150,000 g, and the content of 36-kdalton protein in each P150 and S150 fraction was determined by immunoprecipitation. The percent soluble 36-kdalton is normalized to a combined recovery of 100% in each case (all recoveries were at least 90%).

TABLE IV

Release of the 36-kdalton Protein from P150 Fractions Prepared in Hypotonic TKM Buffer

Extraction of P150*	Percentage of total 36-kdalton protein recovered	
	P150	S150
Resuspension in TKM	84	16
+ 5 mM EDTA/5 mM EGTA	68	32
+ 600 mM NaCl	66	34
+ 1% (wt/vol) Triton X-100	11	89

* P150 pellets, prepared as described in Table I, were resuspended in TKM buffer by repeated aspiration through a 25-gauge needle. The indicated agents were then added and the mixtures were incubated on ice for 20 min. Material remaining particulate was pelleted by centrifugation at 150,000 *g* for 1 h at 0°C.

TABLE V

Effects of Salt and Magnesium on the Solubility of the 36-kdalton Protein

	Homogenate prepared in TK buffer plus:		Homogenate supplemented with:		Distribution of 36-kdalton protein: (percentage)	
	150 mM NaCl	1 mM $MgCl_2$	NaCl (mM)	1 mM $MgCl_2$	P150*	S150
a.	−	+	−	−	97	3
	+	−	−	−	96	4
	+	+	−	−	97	3
b.	−	−	−	−	99	1
	−	−	−	+	98	2
	−	−	150	−	96	4
	−	−	150	+	95	5
	−	−	600	−	53	47
	−	−	600	+	50	50

* P150 fractions here include nuclei.

TABLE III

Distribution of the 36-kdalton Protein from Postnuclear Supernatants Analyzed Directly by Centrifugation into Discontinuous Sucrose Gradients

Fraction	Protein %*	36-kdalton protein		LDH		5′-Nucleotidase		NADH diaphorase	
		%	relative‡ sp act	%	relative sp act	%	relative sp act	%	relative sp act
Postnuclear supernatant after centrifugation	73	6	(0.1)	98.6	(3.2)	<0.2	(<0.01)	21	(0.3)
20% sucrose + 20%/35% interface	14	63	(4.4)	0.6	(0.2)	67	(6.3)	27	(1.3)
35% sucrose + 35%/40% interface	10	26	(2.9)	0.6	(0.2)	30	(2.7)	34	(2.7)
40% sucrose + 40%/50% interface	3	5	(2.9)	0.1	(0.1)	3	(1.2)	18	(3.7)
	(50)§	(40)		(120)		(65)		(52)	

* Percentage of total recovered.
‡ Relative specific activity (postnuclear supernatant = 1.0).
§ Actual recovery of material loaded in the postnuclear supernatant.

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(Table V*b*). Under all these conditions, >95% of the 36-kdalton protein remained particulate. Only the addition of 600 mM NaCl solubilized a significant fraction of the 36-kdalton protein.

When postnuclear supernatants in low salt were centrifuged directly into sucrose gradients (Table III), the presence or absence of 1 mM magnesium in the homogenates made no significant difference in the association of the 36-kdalton protein with light density membranes. When postnuclear supernatants that had been adjusted to 150 mM NaCl were loaded, 70% of the 36-kdalton protein entered the gradients and two-thirds of that was found at the 20%/35% sucrose interface with light density membranes.

These results indicate that the 36-kdalton protein is associated with light density membranes in both low and physiological concentrations of salt, so it is unlikely that weak ionic interactions cause an artefactual association with membranes. The association of 36-kdalton protein with membranes is also found in the presence or absence of added magnesium ions and is maintained with a variety of different homogenization and fractionation procedures. ~50% of the protein can be solubilized by 600–800 mM salt under several conditions. These findings reinforce the conclusion that the 36-kdalton protein is peripherally or indirectly associated with light density membranes.

Immunofluorescent Localization of the 36-kdalton Protein

LOCALIZATION IN UNINFECTED CELLS: To visualize the location of the 36-kdalton protein, we used the anti–36-kdalton serum for indirect immunofluorescent staining of paraformaldehyde-fixed, uninfected CEF. No staining of cells was seen unless fixed cells were permeabilized, indicating that the 36-kdalton protein was not located on the outer cell surface. In fixed cells permeabilized with Triton X-100, a prominent reticular pattern of fluorescence was distributed in a planar fashion throughout the cells (Fig. 3*A*). Neither preimmune serum (Fig. 3*C*) nor anti–36-kdalton serum blocked with purified protein (Fig. 3*D*) gave significant levels of fluorescence. Perinuclear fluorescence was often intense and could be detected in several planes of focus. In addition, reticular staining was excluded from regions of the cell containing actin filaments. To more clearly examine the relative locations of these two structures, we stained 36-kdalton protein indirectly with fluorescein and actin directly with rhodamine-conjugated phalloidin. Phalloidin binds exclusively to polymerized forms of actin (47). The decoration of actin filaments with phalloidin is shown (Fig. 3*B*) in the same cell that is stained with the anti–36-kdalton serum (Fig. 3*A*). Both photographs were taken in the same focal plane and the absence of 36-kdalton staining in areas containing actin filaments is easily seen.

The reticular pattern of 36-kdalton staining was also observed in cells fixed with absolute methanol at −20°C or with 1% glutaraldehyde that was subsequently quenched with sodium borohydride (0.5 mg/ml), although the pattern was finer than that observed after paraformaldehyde fixation. Permeabilization after fixation with either Triton X-100 or acetone gave the same type of reticular staining. Thus, if the reticular pattern is caused by fixation, it occurs by a mechanism common to all of these procedures.

EXTRACTION WITH DETERGENTS: It has been reported that the 36-kdalton protein is associated with an insoluble matrix left when fibroblasts are extracted in situ by nonionic detergent in a hypertonic buffer (10, 13). This hypertonic buffer might have preserved the association of the 36-kdalton protein with a skeletal or membranous structure (4), but it was also possible that the sucrose induced artefactual adsorption of the 36-kdalton protein to detergent-insoluble proteins. We therefore used immunofluorescence to investigate the effects of various detergent treatments on the association of 36-kdalton protein with membranes and the cytoskeleton. Before fixation, we extracted uninfected CEF either with 0.1% Triton X-100 for 1 min or with 0.2% Brij-58 for 5 min in a buffer designed to stabilize the cytoskeleton (PHEM buffer; reference 39). These treatments were chosen because Brij-58 extraction partially removes the plasma membrane and internal membrane-bound organelles without removing the microtrabecular lattice, while Triton X-100 leaves only the open filamentous network of the cytoskeleton (40). Both of these treatments altered the distribution of 36-kdalton staining. Brij-58-extracted cells exhibited intense, patchy aggregations of fluorescence (Fig. 3*E*), while Triton extraction in PHEM buffer reduced the 36-kdalton fluorescence almost to background (Fig. 3*F*). Extraction with Triton for only 10 s resulted in a level of 36-kdalton fluorescence intermediate between the longer Triton and the Brij treatments. Phalloidin staining of actin filaments in cells extracted with these detergents before fixation was unaltered (data not shown). We conclude that the structures that contain the 36-kdalton protein are unstable when the plasma membrane is solubilized by detergent.

COMPARISON WITH ANTI-SPECTRIN STAINING: The pattern of immunofluorescent staining of the 36-kdalton protein is reminiscent of that described for nonerythroid spectrin in fibroblasts (9, 31). To compare the distribution of the 36-kdalton protein with that of spectrin more directly, we stained CEF and a variety of mammalian cells with the anti–36-kdalton serum or with an anti-pig brain spectrin serum (9). The results obtained with CEF are shown in Fig. 4; similar results were obtained with Rat-1, BSC, and NIH 3T3 cells.

FIGURE 3 Immunofluorescent staining of the 36-kdalton protein in uninfected CEF. (*A–D*) CEF growing on glass coverslips were fixed with paraformaldehyde and permeabilized with 0.1% Triton X-100. (*A* and *B*) Cells were treated sequentially with the anti-36-kdalton rabbit serum, with FITC-conjugated second antibody, and with rhodamine-conjugated phalloidin. Cells in the same field were photographed in the same focal plane with barrier filters appropriate to show (*A*) 36-kdalton staining and (*B*) F-actin staining. Arrows indicate a bundle of actin microfilaments (*B*) and the corresponding exclusion of 36-kdalton protein staining (*A*). (*C*) Cells were treated with preimmune rabbit serum and with FITC-conjugated second antibody. (*D*) Cells were treated with anti–36-kdalton serum that had been preincubated with 6.4 μg of purified 36-kdalton protein for 45 min. They were then incubated with FITC-conjugated second antibody. (*E* and *F*) CEF growing on glass coverslips were extracted at room temperature with PHEM buffer containing (*E*) 0.2% Brij-58 (5 min) or (*F*) 0.1% Triton X-100 (1 min) before fixation with paraformaldehyde. The fixed cells were treated with anti-36-kdalton serum and with FITC-conjugated second antibody. Photographs were taken with a 40 × objective (*A*, *B*, and *F*) or with a 63 × oil objective (*C–E*). Bars, 10 μm. × 400 (*A*, *B*, and *F*); × 800 (*C–E*).

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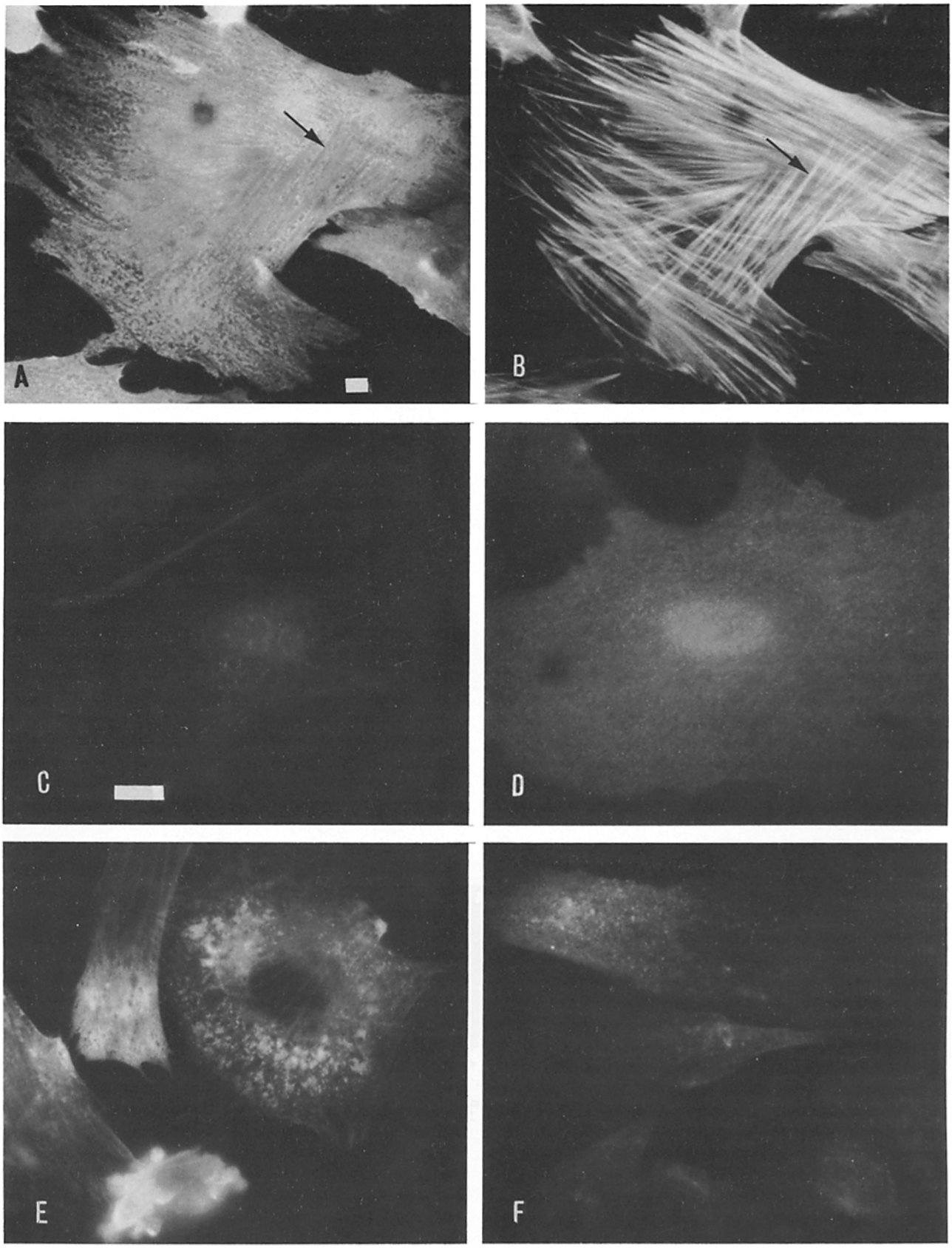

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The staining patterns of spectrin and the 36-kdalton protein were similar, although not identical. Spectrin stained in a somewhat more mottled pattern (Fig. 4*A*) than the reticular pattern of 36-kdalton staining (Fig. 4*C*); both patterns were detected in the same plane of focus as stress fibers (Fig. 4, *B* and *D*). Perinuclear staining of both proteins was evident; this

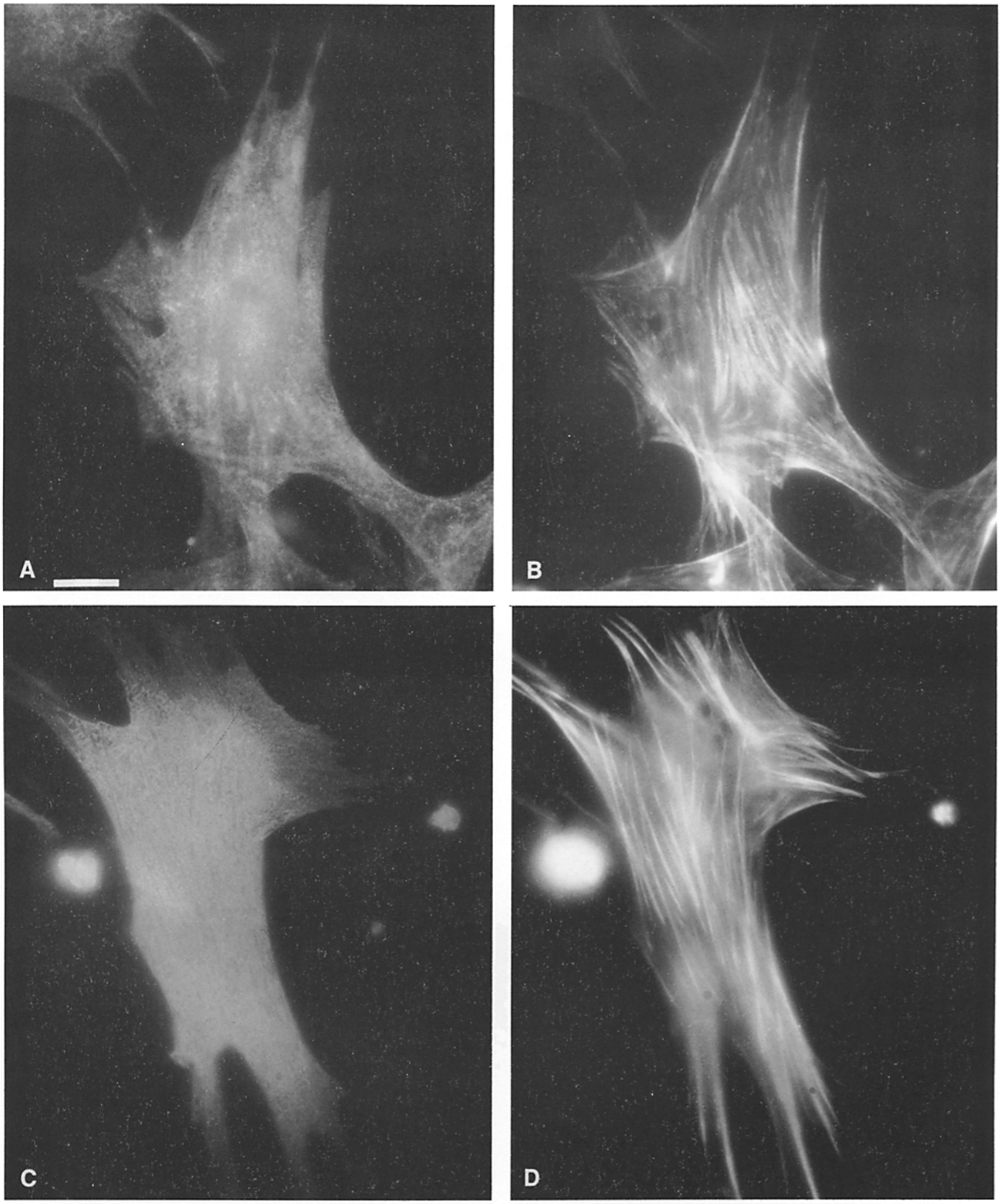

FIGURE 4 Immunofluorescent staining of spectrin in uninfected CEF. Cells were fixed and stained with an anti-brain spectrin antiserum and then phalloidin, or with anti-36-kdalton antiserum and then phalloidin, as described for Fig. 3. Cells in a field were photographed in the same focal plane to show (*A*) spectrin staining and (*B*) F-actin staining, or (*C*) 36-kdalton staining and (*D*) F-actin staining. Cells were photographed with a 63 × oil objective. Bar, 10 μm. × 11,000.

is not apparent in Fig. 4, in which the plane of focus is near the bottom of the cells. Exclusion of both proteins from stress fibers occurred, but the extent of exclusion varied from cell type to cell type. In the mammalian cells, spectrin staining diminished toward the periphery, whereas 36-kdalton protein staining extended completely to the cell margins (data not shown). The similarities between 36-kdalton protein and spectrin staining support the conclusion that the 36-kdalton protein is surface membrane-associated.

LOCALIZATION IN VIRUS-TRANSFORMED CELLS: We examined RSV-transformed cells by immunofluorescence to see whether there was an alteration in 36-kdalton protein distribution in transformed cells. The reticular pattern of 36-kdalton protein fluorescence was clearly evident both in flatter (Fig. 5*A*) and rounder (Fig. 5*C*) cells in the RSV-infected populations. To ensure that the cells we examined were actually transformed, we stained also with rhodamine-conjugated phalloidin to detect actin filaments. The loss of actin

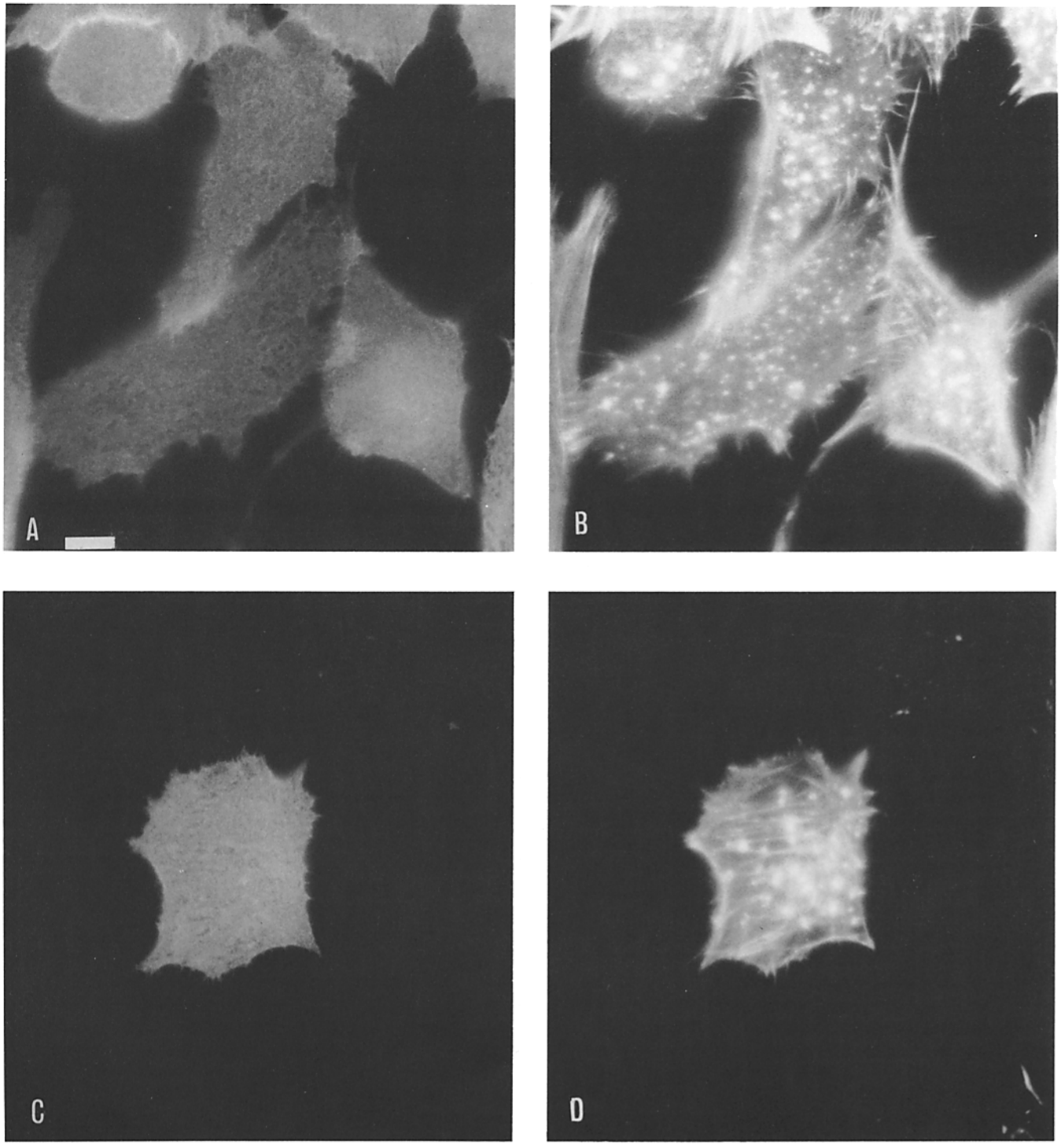

FIGURE 5 Immunofluorescent staining of the 36-kdalton protein in RSV-transformed CEF. Cells transformed with RSV(SR-A) were fixed and stained as described for uninfected cells (Fig. 3, *A* and *B*). Photographs are shown of 36-kdalton immunofluorescence (*A* and *C*) and F-actin fluorescence (*B* and *D*). Relatively flat (*A* and *B*) and rounder (*C* and *D*) cells were photographed with a 63 × objective. Bar, 10 μm. × 800.

cables is a more sensitive criterion of transformation by RSV than is overall cellular morphology (5). Both in flatter (Fig. 5*B*) and in rounder (Fig. 5*D*) cells, significant losses of actin cables had occurred. The punctate areas of bright actin fluorescence probably correspond to the adhesion plaques remaining in these cells (6). Although a few thin actin cables remained in the transformed cells, the exclusion of 36-kdalton protein staining from the area of the cables was no longer detected. In FSV-transformed cells, the reticular pattern of 36-kdalton protein fluorescence was also observed (data not shown). In some of the FSV-infected, fusiform cells where thick actin cables remained, 36-kdalton protein staining was excluded from the areas of the cables. These results indicate that the overall reticular distribution of the 36-kdalton protein is not altered in transformed cells.

DISCUSSION

The results of both fractionation and immunofluorescence analysis indicate that the 36-kdalton protein is primarily located at the periphery of the cell. Several questions are thus raised by these findings. First, with what membrane or cytoskeletal elements is the 36-kdalton protein associated? Second, what is the physical nature of this association? And finally, does the cellular localization of the 36-kdalton protein provide any clues about its possible role in transformation or growth control?

The results of the fractionation experiments presented here demonstrate that the 36-kdalton protein is mainly associated with particulate material and with light density membranes of avian sarcoma virus-transformed CEF. Our results confirm and extend those of Cooper and Hunter (13), who found that both phosphorylated and nonphosphorylated 36-kdalton proteins fractionated mainly with the high speed pellet when cells were homogenized in hypotonic buffer. We have shown in addition that the 36-kdalton protein is associated specifically with light density membranes. Since the bulk of the plasma membranes are present in this fraction, this indicates an association of 36-kdalton protein with plasma membranes, but it is possible that some of the 36-kdalton protein is also associated with smooth endoplasmic reticulum, some of which is also present in light density membranes.

A more detailed picture of 36-kdalton protein localization was obtained by immunofluorescent staining. The reticular pattern of immunofluorescent staining was found in both uninfected and virally transformed fibroblasts. It was more difficult to judge the pattern of staining in very rounded RSV-transformed cells, but nevertheless, reticular staining was apparent. Although the overall distribution of the 36-kdalton protein was unchanged by transformation, we cannot exclude the possibility that a small fraction, for example the 10–20% that is phosphorylated, has an altered location in transformed cells. Intense staining of the abundant protein would probably mask a change in the distribution of a small fraction. Our rabbit antiserum does not discriminate between phosphorylated and nonphosphorylated 36-kdalton polypeptides, so we could not specifically stain either form of the protein.

The presence of the 36-kdalton protein in an extensive, membrane-apposed reticulum is intriguing. This network may interact with the cortex of spectrin and actin that is located on the inside of cell surface membranes. Spectrin in fibroblasts stains by immunofluorescence in a submembranous, patchy, or mottled pattern (9, 31). A network of actin filaments is present inside plasma membranes of fibroblasts in culture (19, 46). As is true for 36-kdalton protein staining, spectrin staining is excluded from areas occupied by stress fibers (9, 31) and is found around nuclei (9). The solubility of the 36-kdalton protein is different than that of spectrin. Whereas the 36-kdalton protein is highly soluble in 1% nonionic detergent and 1 mM EDTA (NP40 lysis buffer) and is at least partially soluble in 0.8 M NaCl, chicken erythrocyte spectrin is insoluble in 1 M KCl, 1% nonionic detergent, and 2 mM EDTA (38). However, such differences in solubility do not preclude an association of 36-kdalton protein with the spectrin matrix.

A possible association of the 36-kdalton protein with nuclei was indicated by results of both fractionation and immunofluorescent staining. We observed that the amount of radiolabeled 36-kdalton protein in nuclear pellets often exceeded that expected if either whole cells or large plasma membrane ghosts were present as contaminants of the nuclear pellets. With immunofluorescent staining, a cagelike network of 36-kalton protein could be seen around some nuclei. (The staining of this network was due specifically to the anti–36-kdalton protein activity of our antiserum.) It is not clear whether this network reflects a specific structure of 36-kdalton protein associated with nuclei, as suggested by the results of cell fractionation, or whether the staining pattern simply reflects an increased amount of cortical cytoplasm in the perinuclear region. Immunofluorescent staining of nuclei isolated by various procedures should help to clarify this issue.

The results of cell fractionation and the similarities with spectrin staining suggest that the 36-kdalton protein may interact with both the plasmalemma and the cytoskeleton. The integrity of the plasmalemma is required for the reticular distribution of the 36-kdalton protein since Brij-58 induced redistribution of the 36-kdalton protein into aggregates and Triton X-100 abolished its fluorescence. The 36-kdalton protein remaining after Brij extraction may be associated with residual fragments of membrane, or perhaps with the cytoskeleton. To further explore the nature of the association of the 36-kdalton protein with membranes, dissociation experiments were performed with salts, chelators, and detergents. The association of 36-kdalton protein with membranes is maintained in low or physiological concentrations of salt and in the presence or absence of added magnesium ions, but is disrupted by the addition of high concentrations of salt, chelators, or nonionic detergents. Cooper and Hunter (13) have also reported that the 36-kdalton protein was particulate in the presence or absence of added magnesium; when EDTA was present, one quarter of the 36-kdalton protein was soluble. The incomplete removal of 36-kdalton protein from particulate material that we observed with chelators or salt suggests that the 36-kdalton protein may be bound to other proteins or the membrane via hydophobic interactions. The ease with which the 36-kdalton protein is solubilized in nonionic detergents supports this idea. The 36-kdalton protein may be directly associated with the plasma membrane as a peripheral protein or may be associated via cortical cytoskeletal proteins; it is not yet possible to distinguish between these alternatives.

The role of the 36-kdalton protein in normal cells is not known. This protein is not present in all types of cells; certain murine B lymphoma cell lines (43) and avian erythroblasts (K. Radke, unpublished data) lack the 36-kdalton protein. Either its function is not required by all cell types, or its function is performed by different proteins in different cell types. The reticular distribution of the 36-kdalton protein and

the similarities between the staining patterns of the 36-kdalton protein and spectrin suggest that the 36-kdalton protein may play a structural role. The spectrin-actin cortex that is located on the inside of erythrocyte membranes contributes structural support to those membranes (8).

It is also not known whether phosphorylation of the 36-kdalton protein is necessary for transformation of fibroblasts by retroviruses whose oncogenes encode tyrosine protein kinases. Its phosphorylation may be necessary but not sufficient for tumorigenesis and for certain features of the transformed phenotype (23, 28, 32). It is interesting that the transforming proteins $pp60^{src}$ (14, 27, 29, 45) and $P140^{gag\text{-}fps}$ (P. Moss, K. Radke, T. Gilmore, and G. S. Martin, submitted for publication) are associated with plasma membranes. Proximity may thus account for the rapidity with which the 36-kdalton protein is phosphorylated after shifting CEF infected by temperature-sensitive mutants of RSV to the permissive temperature (35, 36). Only 10–20% of the 36-kdalton protein is detectable as a phosphoprotein in RSV-transformed cells (37). It will be important to understand how phosphorylation of a fraction of the protein can contribute to changes in cellular phenotype that lead to transformation. If the reticular network of 36-kdalton protein plays a structural role in fibroblasts, and if attachments with other structures are maintained by a fraction of the 36-kdalton polypeptides, then modification of that fraction by phosphorylation could have a significant effect on the organization of the cell cortex.

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