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Purification and Characterization of Zyxin, an 82,000-Dalton Component of Adherens Junctions*

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We describe here the purification and characterization of a recently identified adherens junction protein that has an apparent molecular mass of 82 kDa on sodium dodecyl sulfate-polyacrylamide gels (Beckerle, M. C. (1986) *J. Cell Biol.* 103, 1679–1687). The 82-kDa protein was isolated from avian smooth muscle by a low ionic strength alkaline pH extraction followed by ammonium sulfate fractionation. Sequential chromatographic separation using DEAE-cellulose, phenyl-Sepharose CL-4B, and hydroxylapatite resins results in a purified 82-kDa protein. The 82-kDa protein has a Stokes radius of 5.6 nm and a relative sedimentation coefficient of 3.0 S. The calculated native molecular mass of the protein based on its hydrodynamic properties is 69 kDa, and the derived frictional ratio (f/f_0) is 2.1. The protein does not focus discretely by isoelectric-focusing-sodium dodecyl sulfate-polyacrylamide gel electrophoresis; there are numerous isoelectric point variants in the range of 6.4–7.2, with the average isoelectric point being 6.9. The 82-kDa protein is phosphorylated *in vivo* and appears to be a cytoplasmic component of adherens junctions. The properties of the 82-kDa protein distinguish it from other known adherens junction proteins of this molecular mass. In fibroblasts, the 82-kDa protein is found in adhesion plaques as well as along actin-containing stress fibers near where they terminate at sites of cell-substratum adhesion. It is also found in the cell-cell adherens junctions of pigmented retinal epithelial cells and the dense plaques of smooth muscle cells. Since the 82-kDa protein is found at both cell-substratum and cell-cell adherens junctions, we propose to call it *zyxin*, meaning *a joining*, to indicate that it is found at regions where extracellular ligands are structurally and functionally joined to the cytoskeleton.

Events such as organogenesis and embryogenesis rely upon the ability of cells to adhere to extracellular matrix or other cells. In order to elucidate the molecular mechanism of cell-substratum adhesion, cultured cells displaying very pronounced adhesive structures, called *focal contacts* or *adhesion plaques* (2, 3), are often studied. The ultrastructural organization at the adhesion plaque has been examined (4), and it appears that a transmembrane connection between the extracellular matrix and the cytoskeleton occurs at these areas of the plasma membrane. In particular, bundles of actin filaments terminate at the cytoplasmic face of the membrane at adhesion plaques, while arrays of fibronectin are coincident with the extracellular domain of these substratum contact sites. Integrins, heterodimeric transmembrane receptors for extracellular matrix components, are also found at adhesion plaques (5, 6) where they are thought to be instrumental in coupling the internal cytoskeletal framework with the extracellular environment at sites of adhesion (7, 8). In addition several cytoplasmic components of adhesion plaques, including vinculin, talin, and α-actinin, have been studied extensively and are believed to be important in anchoring actin filaments to the plasma membrane at sites of cell-substratum adhesion (8–14). Nevertheless, the detailed molecular basis by which the actin cytoskeletal framework is directly attached to the membrane at sites of adhesion is currently unresolved, and other molecules are presumably important for the development and regulation of such transmembrane connections.

Recently, we identified an 82,000-dalton adhesion plaque component through the characterization of a nonimmune rabbit serum that stained these structures by indirect immunofluorescence (1). We have now developed a method for the purification of this protein from avian smooth muscle and have characterized a number of its biochemical and biophysical properties. The results of our studies demonstrate that this 82-kDa protein is a previously undescribed component of cell-substratum and cell-cell adherens junctions. Because the 82-kDa protein is localized at sites of extensive transmembrane linkage between extracellular ligands and the cytoskeleton, we propose to call it *zyxin*, from the New Latin combining form *zyxi*, a derivative of the Greek word *zeuxis* meaning *a joining* (42).

MATERIALS AND METHODS

Purification of Zyxin

Extraction of Zyxin from Avian Smooth Muscle—Fresh or frozen chicken gizzards were dissected free of connective tissue and chopped into small pieces. All manipulations were at 4 °C unless otherwise noted. The tissue (300 g) was homogenized in a blender for three successive 10-s bursts in 8.5 volumes of deionized water containing 0.7 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 8,000 × *g* for 10 min in a Sorvall GSA rotor. The supernatant (S1) was discarded, and the pellet (P1) was resuspended briefly in the blender in 8.5 volumes of deionized water containing 0.7 mM phenylmethylsulfonyl fluoride. Centrifugation was repeated (16,000 × *g* for 10 min), and again the supernatant (S2) was discarded. The pellet (P2) was resuspended in 8.5 volumes of buffer A (2 mM Tris-HCl, pH 8.8, 1 mM EGTA,[1] 0.7 mM phenylmethylsulfonyl fluoride, 37 °C), and the pH was adjusted to 9.0 using 2 M NaOH. This suspension was placed in a 37 °C water bath and stirred manually for 45 min. Centrifugation was repeated (16,000 × *g* for 10 min), and the pellet (P3) was discarded. The supernatant (S3) was adjusted to pH

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[1] The abbreviations used are: EGTA, [ethylenebis(oxyethylenenitrilo)] tetraacetic acid; CEF, chicken embryo fibroblast; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography.

7.0 using 0.5 M acetic acid, and 1 M $MgCl_2$ was added to a final concentration of 10 mM. The addition of $MgCl_2$ caused a precipitate to form, and the solution was stirred at room temperature for 15 min. Centrifugation was repeated (16,000 × *g* for 10 min), and the pellet (P4) was discarded. The volume of supernatant (S4) was measured, and 15 g of ammonium sulfate was added per 100 ml, yielding a 26% saturated solution; the suspension was stirred slowly at 4 °C for 45 min. The ammonium sulfate precipitate (P5) was collected by centrifugation (16,000 × *g* for 10 min), and the supernatant (S5) was discarded. The ammonium sulfate pellet was resuspended in a small volume of buffer B-10 (20 mM Tris acetate, pH 7.6, 10 mM NaCl, 0.1% 2-mercaptoethanol, and 0.1 mM EDTA) and dialyzed against buffer B-10 overnight. Samples from each step in the extraction procedure were analyzed by Western immunoblot to determine the efficiency of recovery of zyxin at each step in the protocol. An equivalent percentage of total material from each step in the extraction was resolved on SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with anti-zyxin antibody to reveal the relative amount of the protein present in each fraction. Similarly, the percent recovery of zyxin at each chromatographic step in the purification protocol was determined quantitatively by densitometric analysis of Western immunoblots using a Zeineh scanning densitometer (Bio-Med Instruments Inc.). The relative amount of zyxin present in the final fractions was estimated by comparison with known amounts of bovine serum albumin; samples were resolved by SDS-PAGE, and the silver-stained gels were analyzed by densitometry.

Chromatography—All chromatographic steps were performed at 4 °C. Fractions were collected into plastic tubes, which we found was necessary to prevent nonspecific adsorption of zyxin to the collection vials. All fractions were collected into a protease inhibitor mixture (final concentration, 0.1 mM phenylmethylsulfonyl fluoride; 0.1 mM benzamidine HCl, 1 ng/ml pepstatin A, 1 ng/ml 1,10-phenanthroline) (Sigma). After dialysis, protein contained within the resuspended 26% ammonium sulfate pellet was applied to a DEAE-cellulose (Whatman) column (8.0 × 2.5 cm) equilibrated with buffer B-10. Proteins retained by the column matrix were eluted with a 120-ml linear gradient of 0–150 mM NaCl prepared in buffer B-10. Fractions of 1.25 ml were collected at a flow rate of 0.4 ml/min and were analyzed by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblot to define the fractions containing zyxin. Peak fractions from the DE52 column were pooled, and ammonium sulfate was added to make a 12.5% ammonium sulfate solution. The pooled fractions were then applied to a phenyl-Sepharose CL-4B (Pharmacia LKB Biotechnology Inc.) column (10 × 0.7 cm) equilibrated in buffer B-10 plus 12.5% ammonium sulfate. The column was eluted at a flow rate of 0.3 ml/min with a 20-ml descending linear gradient of 12.5–0% ammonium sulfate prepared in buffer B-10 followed immediately by a 20-ml linear gradient of 0–50% ethylene glycol prepared in buffer B-10. Fractions rich in zyxin, as determined by SDS-PAGE and corresponding Western immunoblot analysis, were pooled. The concentration of ethylene glycol was decreased by adding an equal volume of HAP buffer (1 mM potassium phosphate (mono- and dibasic), pH 7.2, 10 mM NaCl, 0.1 mM EDTA, 0.1% 2-mercaptoethanol) to the pooled fractions. Pooled fractions were applied to a 0.5 ml HPLC-hydroxylapatite (Bio-Rad) column equilibrated in HAP buffer. The column was eluted with a 15-ml linear gradient of 0–75 mM potassium phosphate prepared in HAP buffer. Fractions (0.5 ml) were collected at a flow rate of 0.5 ml/min and analyzed by SDS-PAGE. Proteins were visualized by silver staining (15) to evaluate the purity of zyxin in these fractions.

Determination of the Biophysical and Biochemical Properties of Zyxin

Relative Sedimentation Coefficient—The relative sedimentation coefficient of zyxin was determined by centrifugation through sucrose density gradients (16). Five-ml gradients of 5–20% sucrose (w/v) were prepared at room temperature in buffer B-10 containing 100 mM NaCl. The gradients were cooled to 4 °C, and a 100-μl sample containing partially purified zyxin was layered onto each gradient. The samples were centrifuged at 225,000 × *g* for 14 h in a Beckman SW50.1 rotor. Samples were sedimented through gradients in the presence or absence of standard proteins (Bio-Rad) (myosin (6.4 S), bovine serum albumin (4.3 S), ovalbumin (3.55–3.66 S), and carbonic anhydrase (2.8 S)) (17). Fractions (200 μl) were collected and analyzed by SDS-PAGE and Western immunoblot. The presence of standard proteins in the gradients did not affect the mobility of zyxin significantly; for the sake of accuracy, determination of the relative sedimentation coefficient was performed on samples sedimented in the presence of standard proteins.

Stokes Radius—The Stokes radius of zyxin was estimated by calibrated gel filtration chromatography. A Superose-6 HPLC gel filtration column (Pharmacia) was equilibrated with buffer B-10 containing 100 mM NaCl. The column was calibrated using the following gel filtration standards (Bio-Rad, Sigma): thyroglobulin (8.6 nm), apoferritin (6.06 nm), IgG (5.23 nm), ovalbumin (2.83 nm), and myoglobin (1.91 nm) (18). Samples containing zyxin were applied to the column, and its elution volume relative to standard proteins was determined by SDS-PAGE and Western immunoblot. K_{av} was determined as follows: $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e = elution volume of the solute; V_0 = void volume; and V_t = total gel bed volume. V_0 was determined by chromtography of blue dextran (average M_r = 2,000,000) through the Superose-6 gel matrix.

Isoelectric Point—Two-dimensional gels were performed according to the method of O'Farrell (19). Ampholines (Sigma) in the pH range of 3–10 and 5–8 were used in the first dimension isoelectric focusing gels at concentrations of 30 μl/ml each. Proteins were resolved in the second dimension by 10% SDS-PAGE and were transferred to nitrocellulose for Western immunoblot analysis.

Other Procedures

Antibody Production—BALB/c mice were immunized with purified zyxin according to established methods (20). The first two injections were made with electrophoretically isolated zyxin adsorbed to nitrocellulose. The nitrocellulose was pulverized, and fragments were suspended in Freund's complete adjuvant for the initial injection and Freund's incomplete adjuvant for the second injection. Subsequent immunizations were made with biochemically purified zyxin.

Phase Separation—An extract of embryonic chicken gizzard proteins, purified platelet glycoprotein IIb-IIIa (21), or a mixture of the two samples was prepared in 10 mM Tris HCl, pH 7.4, 150 mM NaCl, and 0.1% precondensed Triton X-114. Phase separation was performed as described previously (22), and the proteins partitioning with the aqueous and detergent phases were analyzed by SDS-PAGE and Western immunoblot.

SDS-PAGE and Western Immunoblot Analysis—Proteins were separated on 10% polyacrylamide gels according to the method of Laemmli (23) using 0.13% bisacrylamide (Bio-Rad). Molecular mass standards (Bio-Rad) were: myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21 kDa), and lysozyme (14 kDa). Western immunoblots were performed by a modification of the procedure developed by Towbin *et al.* (24) as described previously (1). ^{125}I-Protein A (ICN) was used as the secondary reagent.

Immunocytochemistry—Chicken embryo fibroblasts (CEFs) and chicken pigmented retinal epithelial cells were grown on 15-mm coverslips for 24 h and prepared for indirect immunofluorescence as described previously (1). Coverslips were mounted in phosphate-buffered saline containing 50% glycerol and 5 mM *p*-phenylenediamine (Sigma) to reduce photobleaching of rhodamine-conjugated goat anti-mouse antibody (Organon Teknika-Cappell). Gizzards from 40-day-old chickens were fixed for 2 h in 2% paraformaldehyde, placed in 20% sucrose overnight, embedded in OCT compound, and sectioned at −15 °C. Sections 10 μm thick were postfixed for 10 min in 3.7% formaldehyde and prepared for immunofluorescence as above. Micrographs were taken on a Bio-Rad confocal microscope, with an optical scanning thickness of approximately 1 μm.

Immunoprecipitation from ^{32}P-Labeled Cells—Subconfluent CEFs were rinsed three times with phosphate-free Dulbecco's Modified Eagle's Medium and incubated with 1 mCi/ml [^{32}P]orthophosphate (ICN Radiochemicals) for 4 h in phosphate-free medium supplemented with 4% fetal bovine serum (3 ml/60-mm dish). The cells were rinsed in Hanks' balanced salt solution four times, lysed directly in Laemmli sample buffer, and boiled for 5 min. Immunoprecipitations were performed as described previously (25). Autoradiography was performed at −80 °C with Kodak X-Omat AR film with an intensifying screen.

RESULTS

Purification of Zyxin from Avian Smooth Muscle

We recently identified a novel 82-kDa component of chicken embryo fibroblast (CEF) adhesion plaques, hence-

forth referred to as zyxin, by analysis of a nonimmune rabbit serum (previously referred to as F396 serum) (1). In order to identify an appropriate source from which to isolate zyxin, the nonimmune serum was used to probe Western immunoblots of proteins from a variety of embryonic avian tissues. This analysis revealed that although zyxin is ubiquitous it is most abundant by weight in tissues rich in smooth muscle and in fibroblasts (1).

We have now developed a procedure for purifying zyxin from a readily available source of smooth muscle, adult chicken gizzard. Either fresh or frozen tissue may be used; however, we have discovered that the yield of purified zyxin is up to five times greater when fresh gizzards are used. The procedure used for extracting zyxin from avian smooth muscle (Fig. 1*A*) is based largely on the method developed for the purification of α-actinin and vinculin from the same source (26). Briefly, zyxin is isolated from a smooth muscle homogenate by low ionic strength alkaline pH extraction followed by ammonium sulfate fractionation. Samples from various stages in the extraction protocol were analyzed by SDS-PAGE and Western immunoblot analysis using the original nonimmune serum. All detectable zyxin was contained within the 26% ammonium sulfate precipitate (data not shown). It should be noted that zyxin is not abundant in the starting material, and at this stage in the preparation it is difficult to detect as a discrete band on Coomassie blue- or silver-stained SDS-polyacrylamide gels. The presence of zyxin is reliably revealed by Western immunoblot techniques using the nonimmune serum.

Purification of zyxin after ammonium sulfate fractionation was achieved through a series of chromatographic steps. Protein precipitated with 26% ammonium sulfate (Fig. 1*B*, *lane P5*) was passed over a DEAE-cellulose (DE52) column. Zyxin elutes from the DE52 column between 40 and 60 mM NaCl. Peak fractions from the DE52 column were pooled (Fig. 1*B*, *lane D*) and loaded onto a phenyl-Sepharose CL-4B column. Elution of the hydrophobic interaction column with decreasing ammonium sulfate followed by increasing ethylene glycol resulted in substantial purification of zyxin (Fig. 1*B*, *lane P*). No detectable zyxin eluted with decreasing ammonium sulfate; 25–35% ethylene glycol was required to elute the protein from the column. At this point in the purification procedure zyxin can easily be detected on silver-stained SDS-polyacrylamide gels. Fractions containing zyxin were pooled, the concentration of ethylene glycol was decreased, and the sample was applied to an HPLC-hydroxylapatite column. Zyxin eluted from the hydroxylapatite column between 5 and 15 mM potassium phosphate (Fig. 1*B*, *lane H*). Western immunoblot analysis of a parallel gel demonstrates that zyxin isolated by this procedure is recognized by the original nonimmune serum (Fig. 1*C*). By quantitative Western immunoblot analysis, we estimate that the recovery of highly purified zyxin from frozen gizzard is 3–5%, assuming that 100% of the extracted protein is contained within the 26% ammonium sulfate precipitate. The greatest loss of material occurs during DE52 chromatography and results from proteolytic degradation and the fact that the protein elutes from the column in a broad peak. Nevertheless, this step is critical to the purification procedure, and we have been unable to identify an acceptable alternative procedure.

As can be seen in the Western immunoblot shown in Fig. 1*C*, zyxin is susceptible to proteolysis at early steps in the purification. Although protease inhibitors are present throughout the purification procedure, we often detect an immunoreactive polypeptide of approximately 45 kDa by Western immunoblot analysis using anti-zyxin antibody (Fig. 1*C*, *arrowhead*). We have determined that this immunoreactive polypeptide is a proteolytic cleavage product of zyxin; the original nonimmune (F396) serum affinity purified against this electrophoretically isolated polypeptide recognizes an 82-kDa protein by Western immunoblot analysis and also stains adhesion plaques by indirect immunofluorescence (data not shown). In addition this 45-kDa polypeptide accumulates over time.

Zyxin is significantly purified after the chromatographic steps described above; however, in the representative sample shown in Fig. 1*B*, *lane H*, a faintly staining protein migrating at approximately 55 kDa is also visible. We have found that

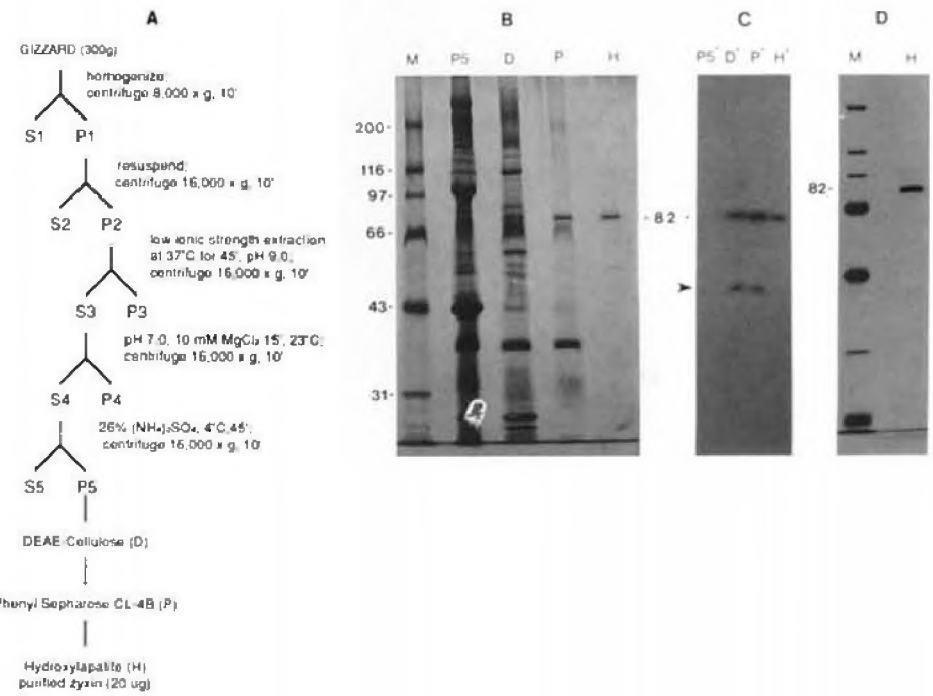

FIG. 1. **Purification of zyxin from avian smooth muscle.** Zyxin is purified from avian smooth muscle as outlined in *panel A*. In *panel B* we present the stages in the purification of zyxin from frozen chicken gizzard since this protein source is readily available to most investigators. Representative samples of protein from each stage in the chromatographic isolation of zyxin are shown in a silver-stained 10% SDS-polyacrylamide gel: *M*, molecular mass standards; *P5*, 26% ammonium sulfate precipitate loaded onto the DEAE-cellulose column; *D*, zyxin-rich fractions from DEAE-cellulose column; *P*, phenyl-Sepharose fractions containing zyxin; *H*, hydroxylapatite fractions containing purified zyxin. *Panel C* shows a Western immunoblot corresponding to the gel in *panel B* and reveals that the purified protein is recognized by the original polyclonal serum. A proteolytic fragment of zyxin which migrates at an apparent molecular mass of 45 kDa is detected in *lanes D'* and *P'* (*arrowhead*). The silver-stained gel shown in *panel D* illustrates that the use of fresh gizzard allows for the purification of zyxin to apparent homogeneity.

we can eliminate all contaminants from the final fractions by selective pooling of fractions at each stage in the preparation, a strategy that reduces the final yield of purified zyxin, or by using fresh gizzard tissue as the starting material (Fig. 1*D*). In preparations starting with 300 g of fresh gizzard, we recover approximately 20 μg of purified protein in the final hydroxylapatite fractions.

Localization of Zyxin at Cell-Substratum and Cell-Cell Adherens Junctions

The original nonimmune (F396) serum that identified zyxin is a low titer reagent that was ineffective for immunocytochemical studies. Consequently, new antibodies directed against purified zyxin were produced in mice. Western immunoblot analysis reveals that the polyclonal mouse serum (M2) specifically recognizes zyxin purified from avian smooth muscle (data not shown) as well as a co-migrating 82-kDa protein from a preparation of total CEF proteins (Fig. 2). These specific antibodies were used to evaluate whether zyxin is a component of cell-substratum and cell-cell adherens junctions. By indirect immunofluorescence of CEFs (Fig. 3*A*), we have determined that zyxin is consistently localized at adhesion plaques and in addition is often present along actin filaments near where they terminate at sites of membrane-substratum adhesion. The extent of co-localization of zyxin along the terminal portion of stress fibers is somewhat variable. In some cells it is present exclusively at the adhesion plaques, whereas in other cells it displays extensive co-localization with actin filaments for some distance away from the adhesion plaques. Interestingly, the entire population of stress fibers within a cell is not labeled with anti-zyxin antibody, indicating that the distribution of the protein is somehow restricted to the ends of the actin filament bundles. The fact that this unusual staining pattern corresponds precisely to that observed with the original nonimmune (F396) serum provides further evidence that the protein we have purified is indeed the antigen recognized by the original nonimmune serum.

Pigmented retinal epithelial cells isolated from chicken embryos exhibit two classes of adherens junctions. In addition to forming focal contacts at their basal surfaces where they interact with the underlying substratum, they form a number of cell-cell contacts including zonulae adherens. These junctional complexes are associated with actin filaments and contain a subset of the proteins identified as adhesion plaque components, including α-actinin and vinculin. In addition to staining adhesion plaques in these cells, anti-zyxin antibody also labels circumferential areas of cell-cell contact at the apical surfaces of pigmented retinal epithelial cells (Fig. 3*B*). It is likely that this staining corresponds to zonulae adherens, since anti-vinculin antibodies also label these peripheral structures in pigmented retinal epithelial cells (data not shown).

In chicken gizzard, the source from which we have purified zyxin, the protein is found in an array of punctate patches at the periphery of individual cells (Fig. 3*C*). This staining pattern corresponds to the distribution of dense plaques, structures that represent the major site of actin-membrane interaction in smooth muscle cells.

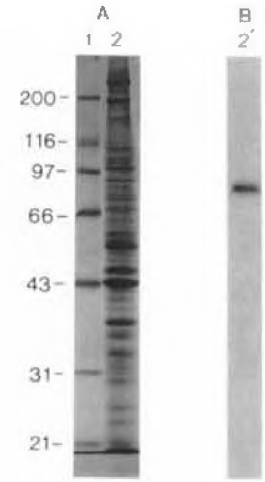

FIG. 2. **Western immunoblot analysis using antiserum generated against purified zyxin.** *A*, a Coomassie blue-stained SDS-polyacrylamide gel showing molecular mass standards (*lane 1*) and total chicken embryo fibroblast proteins (*lane 2*). *B*, the autoradiograph of the corresponding Western immunoblot reveals that the polyclonal antiserum generated against purified zyxin recognizes specifically a polypeptide migrating at an apparent molecular mass of 82 kDa (*lane 2′*). Even upon prolonged exposure of the film, no other immunoreactive polypeptides are detected.

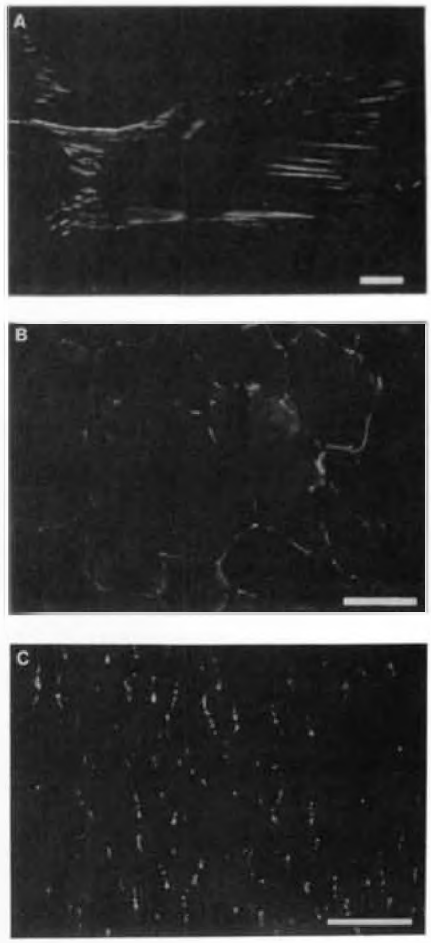

FIG. 3. **Antiserum raised against purified zyxin immunolabels several distinct types of adherens junctions.** *A*, fluorescence micrograph showing the distribution of zyxin in CEFs. Adhesion plaques as well as portions of stress fibers that insert at the membrane at sites of adhesion are stained with this antiserum. *Bar* = 20 μm. *B*, fluorescence micrograph of chicken pigmented retinal epithelial cells showing that zyxin is found in areas of cell-cell contact in this cell type. The antibody labels a circumferential band at the apical surface of each cell. *Bar* = 20 μm. *C*, in smooth muscle cells zyxin is restricted to discrete spots around the perimeter of individual cells, suggesting that the protein is a component of dense plaques. *Bar* = 10 μm.

Biochemical and Biophysical Properties of Zyxin

Stokes Radius—The Stokes radius of zyxin was determined by calibrated gel filtration chromatography on an HPLC

Superose-6 column. Comparison of the elution profile of zyxin with standard proteins having Stokes radii ranging from 1.9 to 8.6 nm reveals that it has a Stokes radius of 5.6 nm ± 0.1 nm (mean ± S.E., $n = 10$) (Fig. 4). Interestingly, zyxin elutes from the column with a Stokes radius greater than that expected of a globular protein of 82,000 daltons. The Stokes radius of zyxin is not affected by high salt (0.5 M NaCl or 0.5 M KI) or detergent (1.0% Triton X-100).

Relative Sedimentation Coefficient—The relative sedimentation coefficient of zyxin was estimated by sucrose density gradient centrifugation. Analysis of four separate gradients from three independent trials comparing the extent of migration of zyxin through the gradient against the migration of internal standard proteins reveals that it has a relative sedimentation coefficient of 3.0 S ± 0.2 S (mean ± S.E., $n = 4$) (Fig. 5). This value is lower than expected for a globular molecule of 82,000 daltons.

Based on these experimentally determined properties, we have estimated the native molecular weight of zyxin to be 69,000 by the method of Siegel and Monty (27) in which $M = (6\pi\eta asN)/(1 - \bar{v}\rho)$ (where η = viscosity, a = Stokes radius, s = sedimentation coefficient, N = Avogadro's number, $\bar{v}$ = partial specific volume, ρ = density). We have assumed a partial specific volume of 0.725 cm^3/g (16). The result of this analysis suggests that the protein is monomeric under our experimental conditions. The calculated frictional ratio ($f/f_0 = a/(3\bar{v}M/4\pi N)^{1/3}$ where M = molecular weight) of zyxin is 2.1, indicating that the protein is an asymmetric molecule.

Isoelectric Point—Samples containing zyxin isolated from avian smooth muscle were resolved by two-dimensional gel electrophoresis. From analysis of 14 separate two-dimensional gels from four independent isoelectric focusing-SDS-PAGE experiments, we have established that zyxin has an average isoelectric point of 6.9. Fig. 6 shows a representative Western immunoblot used to determine the isoelectric point of zyxin. By this method, the protein exhibits substantial isoelectric point heterogeneity with numerous discrete isoforms detectable between pH 6.4 and 7.2

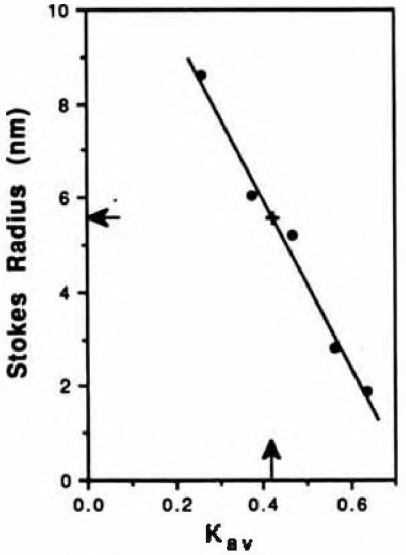

FIG. 4. **Determination of the Stokes radius of zyxin.** Sample containing zyxin was applied to a calibrated HPLC Superose-6 gel filtration column. Comparison of proteins with known Stokes radii (thyroglobulin (8.6 nm), apoferritin (6.06 nm), IgG (5.23 nm), ovalbumin (2.83 nm), and myoglobin (1.91 nm)) chromatographed under the same conditions allowed us to estimate a Stokes radius of 5.6 nm ± 0.1 nm (mean ± S.E., $r^2 = 0.98$) for zyxin. The *arrow* along the *horizontal axis* indicates K_{av} (0.42) for zyxin.

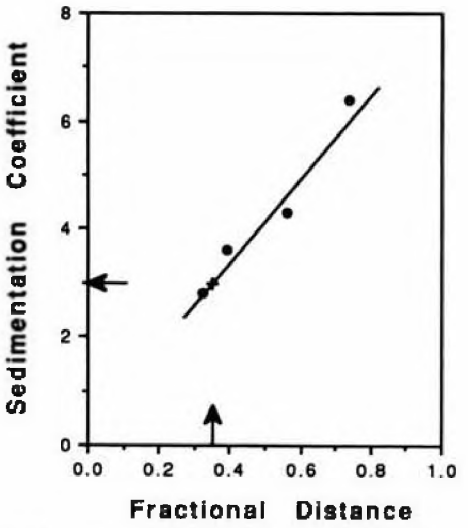

FIG. 5. **Estimation of the relative sedimentation coefficient of zyxin.** Sample containing zyxin plus standard proteins was centrifuged through a 5–20% sucrose gradient. Sedimentation coefficient values for the standard proteins are as follows: myosin (6.4 S), bovine serum albumin (4.3 S), ovalbumin (3.55–3.66 S), carbonic anhydrase (2.8 S). The *arrow* along the *horizontal axis* marks the fractional distance (0.35) the protein migrated through the gradient. The relative sedimentation coefficient of zyxin is 3.0 S ± 0.2 S (mean ± S.E., $r^2 = 0.97$).

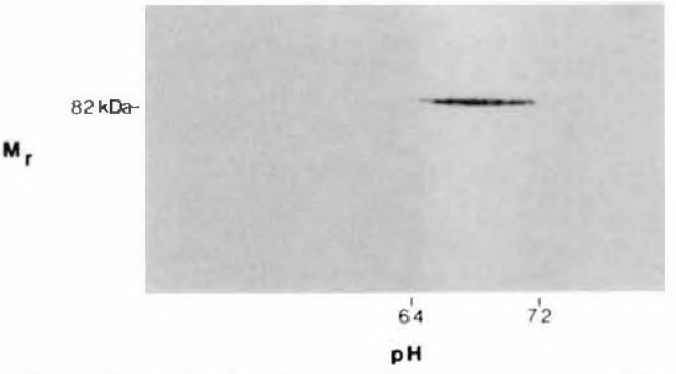

FIG. 6. **Determination of the isoelectric point of zyxin.** The isoelectric point of zyxin was determined by two-dimensional gel electrophoresis. Zyxin has an average pI of 6.9 as determined by Western immunoblot analysis using anti-zyxin antibody followed by ^{125}I-protein A. The protein exhibits substantial heterogeneity in the isoelectric focusing dimension; a number of discrete isoelectric point variants ranging from 6.4 to 7.2 are visible in the autoradiograph.

Since phosphorylation is a common post-translational modification that contributes to isoelectric point heterogeneity, we have examined the possibility that zyxin is phosphorylated *in vivo*. Immunoprecipitation of zyxin from ^{32}P-labeled CEFs reveals a prominently labeled 82-kDa phosphoprotein (Fig. 7, *lane 2*). Control, normal mouse serum does not immunoprecipitate a phosphoprotein in the molecular weight range of zyxin (Fig. 7, *lane 3*). Although we have not yet performed phosphoamino acid analysis on *in vivo* phosphorylated protein, we have observed that a limited amount of the phosphate associated with the immunoprecipitated protein is alkali insensitive, making it a candidate for a phosphotyrosine-containing protein (28).

Subcellular Compartment—The adhesion plaque can be viewed as consisting of extracellular, membrane, and cytoplasmic domains. We have undertaken several approaches to determine whether or not zyxin has the properties expected of an extracellular or transmembrane protein. Evidence that

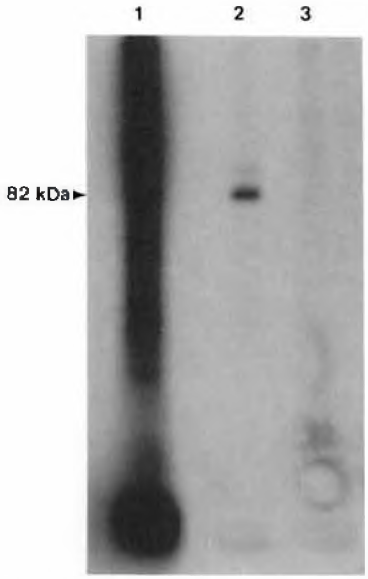

FIG. 7. **Zyxin is phosphorylated *in vivo*.** Autoradiograph showing total ^{32}P-labeled CEF proteins; 3-h exposure of 0.4% of total labeled protein (*lane 1*). Quantitative immunoprecipitation using anti-zyxin antisera (*lane 2*); a phosphorylated protein is apparent at 82,000 daltons; 36-h exposure, 50% of total immunoprecipitated protein was loaded onto the gel. Immunoprecipitation using a control normal mouse serum; 36-h exposure of sample prepared as for *lane 2* (*lane 3*).

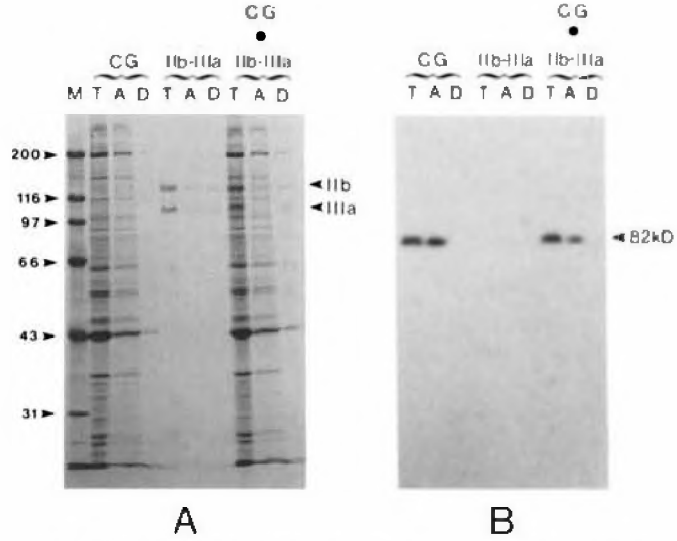

FIG. 8. **Triton X-114 phase separation.** *A*, Coomassie blue-stained 10% SDS-polyacrylamide gel showing three distinct phase separation experiments: chicken gizzard proteins alone (*CG*), glycoprotein IIb-IIIa alone (*IIb-IIIa*), and a mixing experiment (*CG + IIb-IIIa*). *M*, molecular mass standards; *T*, total protein; *A*, aqueous phase; *D*, detergent phase. The transmembrane glycoprotein IIb-IIIa was used to demonstrate partitioning of an amphiphilic protein to the detergent phase. Notice that approximately half of IIb-IIIa is found in the detergent phase. *B*, corresponding Western immunoblot reveals that zyxin is found exclusively in the aqueous phase.

zyxin is not an extracellular or transmembrane protein comes chiefly from three studies. First, detection of the protein by indirect immunofluorescence requires detergent permeabilization of the cell, indicating that all antigenic determinants are intracellular. Second, extraction of the protein from smooth muscle is not enhanced in the presence of detergent. Concentrations of 0.1–1.0% Triton X-100 have no effect on the amount of zyxin recovered from the homogenized tissue. Finally, in Triton X-114 phase separation experiments, zyxin does not exhibit properties expected of an integral membrane protein. In this approach, a solution of the nonionic detergent, Triton X-114, containing the proteins to be analyzed is subjected to a temperature-induced phase separation (22). Proteins partition into either the aqueous or detergent phase depending on their properties; amphiphilic proteins partition with the detergent phase whereas hydrophilic proteins associate with the aqueous phase. Zyxin is found exclusively in the aqueous phase (Fig. 8), suggesting that it does not contain extensive regions of hydrophobic amino acids and therefore is not likely to be a transmembrane protein. Taken together, these observations suggest that zyxin is a cytoplasmic component of adhesion plaques. Furthermore, we have also examined whether zyxin binds concanavalin A by lectin-affinity chromatography and have detected no interaction (data not shown).

Since zyxin is found both in the adhesion plaque proper as well as along actin filaments near where they associate with the membrane, we have investigated the possibility that it binds directly to filamentous actin. We have not detected a direct interaction between zyxin and filamentous actin by sedimentation assays although the known actin-binding protein, α-actinin, does co-sediment with actin filaments under the assay conditions we utilized (data not shown).

DISCUSSION

In this paper, we describe a procedure for the purification of a low abundance 82,000-dalton adherens junction protein from adult chicken gizzard. The 82-kDa protein, zyxin, is purified to apparent homogeneity by this procedure as evaluated by silver staining of SDS-polyacrylamide gels. Although by one-dimensional SDS-PAGE we cannot exclude the possibility that more than one species of protein is migrating at 82 kDa this seems unlikely given the results of Western immunoblots of two-dimensional gels in which both the nonimmune rabbit serum and antibodies raised against purified zyxin recognize the same cluster of protein spots (data not shown). Indirect immunofluorescence using antibodies directed against purified zyxin reveals that it is present at sites of cell-substratum and cell-cell adhesion in a variety of cells.

In addition, we have characterized a number of the biochemical features of zyxin. The protein appears to be cytoplasmic and is relatively insoluble at neutral pH, having an isoelectric point of 6.9. The hydrodynamic properties of zyxin suggest that it is an asymmetric molecule and exists as a monomer at physiological ionic strength. Zyxin is phosphorylated *in vivo*, a finding consistent with the numerous isoelectric point variants we have observed by isoelectric focusing-SDS-PAGE.

One important issue to resolve is that of whether zyxin is related to previously identified adherens junction components. Although a number of other proteins with molecular masses in the range of 82,000 daltons have been identified previously as adhesion plaque components, including protein kinase C (29), the calcium-dependent protease type II (30), and a 65–76-kDa phosphoprotein termed paxillin (31, 32), these proteins appear to be distinct from zyxin on the basis of several criteria, including biochemical properties, tissue distribution, and/or subcellular localization. For example, protein kinase C isolated from rat liver exhibits biochemical properties that are quite distinct from those we have defined for the avian 82-kDa protein; the rat liver enzyme has a Stokes radius of 4.2 nm, sedimentation coefficient of 5.1 S, and pI of 5.6 (33). Furthermore, protein kinase C is enriched in brain, a tissue in which zyxin is difficult to detect. Similarly,

the 80-kDa large subunit of the calcium-dependent protease type II found in adhesion plaques has been isolated from chicken gizzard (34) and exhibits biochemical and biophysical properties that distinguish it from zyxin. In addition, the large subunit of calcium-dependent protease type II routinely co-purifies with a smaller 30-kDa polypeptide which we do not observe in our preparations of zyxin. Moreover, calcium-dependent protease type II is not detected by immunological means in zonulae adherens (30), cell-cell adherens junctions that appear to contain zyxin. Likewise, the recently identified phosphoprotein paxillin also appears to be excluded from areas of cell-cell contact (32). Although paxillin has a reported molecular weight similar to that of zyxin, the two proteins do not co-migrate on SDS-polyacrylamide gels.[2]

An 82-kDa protein present in zonulae adherens has been identified recently (35); however, this protein, referred to as radixin, has not been detected in focal contacts, suggesting that it is not identical to the 82-kDa protein we have isolated. Because the physical properties of radixin have not yet been characterized, we are unable to make comparisons based on these criteria. However, preliminary immunological data suggest that radixin is distinct from zyxin.[3]

A human autoimmune serum that recognizes a component of adhesion plaques by indirect immunofluorescence has also been described (36). This autoimmune serum recognizes 73- and 80-kDa proteins in cultured PtK2 cells and 53 kDa proteins in both HEp-2 and baby hamster kidney-21 cell lines by Western immunoblot analysis but does not recognize an avian antigen (*i.e.* zyxin) by either indirect immunofluorescence or Western immunoblot analysis.[4] Therefore, it is difficult to establish the relationship between zyxin and the antigens recognized by the human autoimmune serum.

Zyxin also appears distinct from the phosphoprotein ezrin (p81), a cytoskeletal protein identified in microvilli and other types of cell surface projections that is closely related to cytovillin (37–40). Many of the characteristics of ezrin, including its Stokes radius, sedimentation coefficient, and hydroxylapatite-binding properties (40, 41) distinguish it from the 82-kDa protein we have isolated. Perhaps most significantly, ezrin has not been found at adhesion plaques although its distribution in fibroblastic cells has been examined in some detail (40, 41).

In summary, we have purified an 82-kDa component of adherens junctions from avian smooth muscle. We believe that zyxin is a previously uncharacterized component of adherens junctions for the following reasons. 1) Zyxin is immunologically distinct from other adherens junction components. 2) The biochemical and biophysical properties of zyxin are significantly different from other adherens junction components of similar molecular mass. 3) The distribution of zyxin at sites of cell-substratum adhesion is unusual in that the protein is found at focal contacts as well as along stress fibers near where they terminate at the plasma membrane. Although zyxin appears distinct from other adherens junction proteins we still have little insight into its role at these dynamic structures. However, its subcellular location puts it in a position where it could be part of the machinery responsible for linking actin filaments to the plasma membrane at sites of cell-substratum adhesion. The purification and characterization of zyxin are a first step toward defining the function of this protein at sites of cell-cell and cell-substratum adhesion.

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[2] C. E. Turner, J. R. Glenney, and K. Burridge, personal communication.

[3] M. C. Beckerle and S. Tsukita, unpublished observations.

[4] J.-L. Senecal, A. Crawford, and M. C. Beckerle, unpublished observations.

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