The Two Major Membrane Skeletal Proteins (Articulins) of *Euglena gracilis* Define a Novel Class of Cytoskeletal Proteins

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Abstract. 60% of the peripheral membrane skeleton of *Euglena gracilis* consists of equimolar amounts of two proteins (articulins) with M_rs in SDS gels of 80 and 86 kD. To understand eventually how these proteins assemble and function in maintaining cell form and membrane integrity we have undertaken a molecular characterization of articulins. A λgt11 expression library constructed from *Euglena gracilis* mRNAs was screened with antibodies against both articulins. Two sets of cDNAs were recovered, and evidence from three independent assays confirmed that both sets encoded articulins: (*a*) Anti-articulin antibodies recognized a high molecular weight β-galactosidase (β-gal) fusion protein expressed in bacteria infected with λgt11 cDNA clones. (*b*) Antibodies generated against the bacterially expressed β-gal fusion protein identified one or the other articulin in Western blots of *Euglena* proteins. These antibodies also localized to the membrane skeletal region in thin sections of *Euglena*. (*c*) Peptide maps of the β-gal fusion protein were similar to peptide maps of *Euglena* articulins. From the nucleotide sequence of the two sets of cDNAs an open reading frame for each articulin was deduced. In addition to 37% amino acid identity and overall structural similarity, both articulins exhibited a long core domain consisting of over 30 12-amino acid repeats with the consensus VPVPV---V---. Homology plots comparing the same or different articulins revealed larger, less regular repeats in the core domain that coincided with predicted turns in extended β-sheets. Outside the core domain a short hydrophobic region containing four seven-amino acid repeats (consensus: APVTYGA) was identified near the carboxy terminus of the 80-kD articulin, but near the amino terminus of the 86-kD articulin. No extensive sequence similarities were found between articulins and other protein sequences in various databanks. We conclude that the two articulins are related members of a new class of membrane cytoskeletal proteins.

THE membrane skeleton of higher eukaryotes is required for a number of important cell functions including the maintenance of cell shape, the stabilization of the plasma membrane (reviewed in Elgsaeter et al., 1986; Bennett, 1990), generating and maintaining plasma membrane domains (Rodriguez-Boulan and Nelson, 1989), and lymphocyte activation (Lee et al., 1988). The most abundant membrane skeletal proteins of higher eukaryotes are the spectrins (or their nonerythroid homologues, fodrins). Spectrins are rod-shaped proteins which form heterodimers from large (M_r = 240 and 260 kD) α and β subunits. The spectrin subunits have similar overall structures, but each is encoded by a different gene (Moon and McMahon, 1990; Sahr et al., 1990). Another high molecular mass protein, ankyrin, links spectrin tetramers $(\alpha\beta)_2$ to integral membrane proteins, including the anion transporter in the erythrocyte (Hargreaves et al., 1980), Na^+, K^+-ATPase in epithelial cells (Nelson and Veshnock, 1987; Nelson and Hammerton,

1989; Morrow et al., 1989) and the voltage-gated sodium channel in neurons (Srinivasan et al., 1988). Spectrin is an actin binding protein, and the spectrin tetramers are interconnected by short actin oligomers to form a planar meshwork (reviewed in Bennett, 1990). The spectrin-based membrane skeleton is well documented and well characterized; the peripheral membrane skeleton of *Euglena gracilis* also maintains cell form, but lacks spectrin and is organized differently (reviewed in Dubreuil et al., 1992).

In *Euglena* the membrane skeleton is organized into ∼40 narrow, ribbon-like strips that overlap (articulate) laterally to generate the pronounced ridges and grooves diagnostic of these cells. In the region of overlap a group of microtubules is arranged parallel with and connected by filamentous bridges to the strips. In some genera these strips must be able to slide relative to one another (possibly microtubule motor driven) to accommodate the rapid shape changes known as euglenoid movements (Suzuki and Williamson, 1985). Microtubules, however, do not appear to stabilize surface form. It is other proteins of the membrane skeleton that maintain both cell surface and whole cell form (reviewed in Dubreuil et al., 1992).

The most abundant proteins of the *Euglena* membrane skeleton are the equimolar articulins (M_r = 80 and 86; Dubreuil and Bouck, 1985). The articulins appear to form hetero-oligomers that when solubilized by urea or NaOH can reassemble on NaOH stripped membranes (Dubreuil and Bouck, 1988). Articulins interact with the plasma membrane through the noncovalent binding of the 80-kD articulin to the major integral plasma membrane protein IP39 (Rosiere et al., 1990). Actin is not present in the membrane skeleton, and the microtubules found in vivo (Leedale, 1964; Mignot, 1965; Dubreuil and Bouck, 1985) are not required for in vitro assembly of the membrane skeleton.

To determine the relationship of the articulins to one another and to other cytoskeletal proteins, we have selected clones encoding articulins from a *Euglena* cDNA expression library using antibody probes. Primary sequence analysis revealed that both articulins have a distinct tripartite organization that consists of amino- and carboxy-terminal regions separated by a central core domain made up of 33 12-amino acid repeats. The greatest amino acid similarity occurs in the core domain and in four copies of a hydrophobic seven amino acid repeat found at opposite ends of the two proteins. The overall sequence similarity together with the biochemical evidence indicates that the articulins are related proteins, but probably are encoded by different genes. The articulins have no significant similarity to any previously sequenced proteins, suggesting that they are representatives of a new class of cytoskeletal proteins.

Materials and Methods

Cell Culture and Surface Isolation

Euglena gracilis strain Z was cultured axenically to densities of ~5 × 10⁵ cells/ml, and harvested by centrifugation. Surface isolates, consisting of the plasma membrane, the underlying membrane skeleton, microtubules and bridgework were prepared and enriched on sucrose gradients as previously detailed (Dubreuil and Bouck, 1985).

Partial Purification of the 80- and 86-kD Articulins

Surface isolates were extracted with a solution containing 4 M urea, 10 mM Tris-HCl, and 5 mM EDTA (4 M urea) for 15 min at 4°C, and the soluble and insoluble fractions were separated in a microfuge at 12,500 g for 10 minutes. The urea soluble (peripheral) proteins were stored on ice and the remaining urea resistant proteins were extracted by resuspending the pellet in 20 mM NaOH and incubating for 15 min at 4°C. After centrifugation at 12,500 g for 10 min, the soluble proteins were removed and the NaOH stripped plasma membranes were resuspended in a solution of 10 mM Hepes at pH 7.0, 25 mM KCl and 0.05% NaN₃. The urea soluble fraction and the NaOH stripped membranes were combined and dialyzed against a solution containing 10 mM sodium phosphate at pH 8.0, 25 mM KCl and 0.1% β-mercaptoethanol for 60–90 min at 4°C (Dubreuil and Bouck, 1988). Proteins that reassembled on the plasma membrane (mostly articulins) were sedimented by centrifugation at 12,500 g for 10 min. Treatment with 4 M urea for 15 min at 4°C resolubilized the bound proteins which were then separated from the plasma membranes by centrifugation. The soluble fraction is referred to as the membrane affinity-enriched articulins.

Phenyl Sepharose (Sigma Chem. Co., St. Louis, MO) columns (2 ml bed volume) were prepared and equilibrated at room temperature with 10 vol of 2 M NaCl in 4 M urea extraction buffer. Approximately 300 μl of the membrane affinity–enriched articulins made 2 M in NaCl were loaded on the column at room temperature, followed by washing with 5 vol of 2 M NaCl in 4 M urea extraction buffer. The column was eluted with 1 M NaCl in 4 M urea extraction buffer, thereby releasing proteins bound by hydrophobic interactions at high ionic strength (Rosengren et al., 1975) as a single peak. The latter proteins are referred to as phenyl Sepharose–purified articulins.

One- and Two-Dimensional Gel Electrophoresis

SDS-PAGE was carried out as described (Laemmli, 1970, as modified by Dubreuil et al., 1988). Proteins analyzed in two dimensions were first separated by nonequilibrium pH gradient electrophoresis (NEpHGE; O'Farrel et al., 1977) in capillary tubes (Garrels, 1979), followed by SDS-PAGE.

Peptide Mapping

Polypeptides were separated by SDS-PAGE, individual bands excised and then radioiodinated with chloramine T (Sigma Chem. Co.) as the catalyst. After washing in 20% methanol, the polypeptides were digested with TPCK-treated trypsin or with TLCK-treated α-chymotrypsin (Worthington Biochemical Corp., Freehold, NJ; Elder et al., 1977; Granger and Lazarides, 1984). Soluble peptides were lyophilized, resuspended in 20% acetic acid, and chromatographed in two dimensions on silica G-25 plates (Brinkmann Instruments, Inc., Westbury, NY; Dubreuil et al., 1988).

Monoclonal and Polyclonal Antibodies

Hybridomas secreting mAbs were generated as described (Dubreuil and Bouck, 1988) from mice immunized with membrane affinity–enriched articulins. mAb isotyping (courtesy of Dr. Thom Rosiere) using a kit (Hyclone Laboratories, Logan, UT) showed that mAb 3G1 was an IgG₃.

Polyclonal antisera were generated against protein bands excised from preparative SDS polyacrylamide gels; the gel slices were equilibrated in a solution of 10 mM sodium phosphate at pH 7.8 with 0.15 M NaCl (PBS) and homogenized. An equal volume of Freund's adjuvant was added to the homogenate, and the mixture was emulsified. Female white New Zealand rabbits were injected subcutaneously at multiple sites along the back, followed by booster injections at day 30 and day 60. Blood was collected from the ear vein before the primary injection (preimmune) and 1 wk after the final immunization (modified from Granger and Lazarides, 1984). Serum was collected, clarified by centrifugation, and stored at 4°C in 0.05% NaN₃. The antisera raised against the two articulins, designated as 80pp-2 and 86pp-2, were affinity purified as follows. Articulins were separated on SDS gels, transferred to nitrocellulose filters, and the individual bands separately excised. The nitrocellulose filter was blocked with a solution of 100 mM Tris-HCl at pH 8.0, 0.15 M NaCl and 0.1% Tween-20 (TBST) with 1% BSA. After several washes with TBST and one with TBS (no Tween-20) bound antibodies were eluted with 1.05 ml of 10% p-dioxane in 0.2 M glycine-HCl at pH 2.8. The eluted antibodies were neutralized with 525 μl of 1 M K₂HPO₄ and 315 μl TBS containing 1% BSA. The nitrocellulose filters were washed with 315 μl TBS and this was pooled with the neutralized antibodies. The antibodies were rinsed and concentrated with a Centricon 30 filtration unit (Amicon, Danvers, MA).

Immunoelectron Microscopy

Cells were sedimented and fixed in 0.5% glutaraldehyde for 10 min at room temperature. During dehydration in ethanol the temperature was lowered to −20°C. Embedding in Lowicryl was carried out at −20°C for 7 d. Sections were mounted on carbon stabilized films and incubated with primary and secondary antibody (goat antirabbit conjugated to 15-nm gold particles. AuroProbe EM GAR G15; Amersham). After rinsing in PBS, the sections were fixed in 1% glutaraldehyde, rinsed and dried, and examined in a JEOL 100EX electron microscope.

Screening the cDNA Library

Approximately 5 × 10⁵ pfu from a λgt11 *Euglena gracilis* cDNA library (Levasseur et al., submitted) were plated and lifted onto nitrocellulose filter discs as described (Huynh et al., 1985). The discs were washed twice with 100 mM Tris-HCl at pH 8.0, 0.15 M NaCl, 0.05% NaN₃, and 0.1% Tween-20 (TBSNT), and then twice with BLOTTO (5% lowfat dry milk) in TBSNT for 30 min. The affinity-purified polyclonal antisera 80pp-2 and 86pp-2 raised against the 80- and 86-kd articulin, respectively, were combined in BLOTTO/TBSNT and incubated with the plaque lifts for 60 min at room temperature. After five rinses in BLOTTO/TBSNT the discs were incubated with alkaline phosphatase conjugated to goat antirabbit in BLOTTO/TBSNT. After extensive washing successively in BLOTTO, TBSNT, and 150 mM Tris-HCl, pH 8.8, a chromogenic substrate solution (60 μg/ml nitroblue tetrazolium and 60 μg/ml 5-bromo-4-chloro-3-indoyl phosphate in 150 mM Tris-HCl at pH 8.8) was added. Positive plaques in the lifts were cored from the master plates and rescreened at least three times or until all plaques were immunoreactive.

Lambda DNA was purified using LambdaSorb (Promega, Madison, WI) and subcloned into M13 and Bluescribe (Stratagene, La Jolla, CA) vectors. Plasmid preparations, subcloning, and agarose gel electrophoresis followed published procedures (Maniatis et al., 1982; Sambrook et al., 1989).

RNA Blotting

Total RNA from *Euglena gracilis* (CsCl pellet; Levasseur, P. J., Q. Meng, and G. B. Bouck, manuscript submitted for publication) was separated by electrophoresis in a 1% agarose gel containing 10 mM sodium phosphate at pH 7.4 and 1 M formaldehyde. RNA samples (15 μg) were loaded in a solution containing 10 mM sodium phosphate at pH 7.4, 50% formamide, 1 M formaldehyde, 10% glycerol, 8 mM EDTA, 0.12% SDS, 1 mg/ml bromophenol blue and 1 mg/ml xylene cyanol. The running buffer contained 10 mM sodium phosphate, pH 7.4, and 1 M formaldehyde. After electrophoresis the RNA was transferred to GeneScreen (DuPont Co., Wilmington, DE) using the manufacturer's protocol. Prehybridization, hybridization, probe preparation, washing, and detection were carried out as described (Kaufman et al., 1985).

Preparation of Lysogenic Strains and Lysogen Extracts

β-galactosidase (β-gal) fusion proteins were prepared from Y1089 *E. coli* infected with λgt11 clones 80/4 and 86/5 at a multiplicity of infection of 100 (Huynh et al., 1985). After incubation at 30°C for 20 min, the Y1089 cells were streaked on LB/ampicillin plates, incubated overnight at 30°C, and replica tested on LB/ampicillin plates at 30° and 42°C. Lysogens (which showed little growth at 42°C) were selected from the 30°C plates and maintained as colonies on LB/ampicillin.

Lysogen extracts containing the β-gal fusion proteins were prepared by inoculating lysogenic colonies in 5 ml of LB/ampicillin and growing overnight at 30°C. After dilution 100-fold in 5 ml of fresh LB/ampicillin medium the cells were incubated for 2 h at 30°C with shaking followed by addition of IPTG to a final concentration of 10 mM (IPTG was omitted for controls), and incubation for 15 min at 45°C. Cultures were then shaken for 2 h at 37°C. 1.5-ml aliquots were centrifuged at 12,500 *g*, the supernatant was discarded, and the pellets were disrupted by brief vortexing. The pellets were resuspended in 100 μl of hot SDS-PAGE sample buffer, vortexed for 1 min, placed in a boiling water bath for 5 min, and vortexed again. The extract was clarified by centrifugation for 4 min at 12,500 *g* and loaded on SDS polyacrylamide gels. The presence of a high molecular weight Coomassie blue staining band in the IPTG-induced sample signaled the presence of the β-gal fusion protein. Specific antibodies against the articulins further verified the identity of the fusion proteins in Western blots.

DNA Sequencing

Nested deletions of the cDNAs in M13mp18 were prepared with Exo III (Erase-a-base; Promega) as recommended by the manufacturer. Single-strand DNA sequencing was carried out by the dideoxy method using Sequenase (United States Biochemical Corp., Cleveland, OH), using the universal primer. In addition, some primers were synthesized (Laboratory for Molecular Biology, University of Illinois at Chicago, DNA synthesis facility) for regions not spanned in the deletions. Sequenase was also used for double-strand DNA sequencing following the procedures in Sambrook et al. (1989).

Results

The Articulins Are Related but Distinct Proteins

The 80- and 86-kD articulins reassembled stoichiometrically (1:1) on NaOH stripped membranes in reassociation experiments (Dubreuil and Bouck, 1988), thereby generating a method useful for affinity enrichment of these proteins in the present study. The membrane affinity–enriched fraction was further purified by hydrophobic interactions on phenyl Sepharose columns, yielding a nearly homogeneous mixture of the two proteins (data not shown). Protein–protein interactions may account in part for the fact that the articulins copurify through these procedures, but each seemed to bind to phenyl Sepharose independently as they could be partially
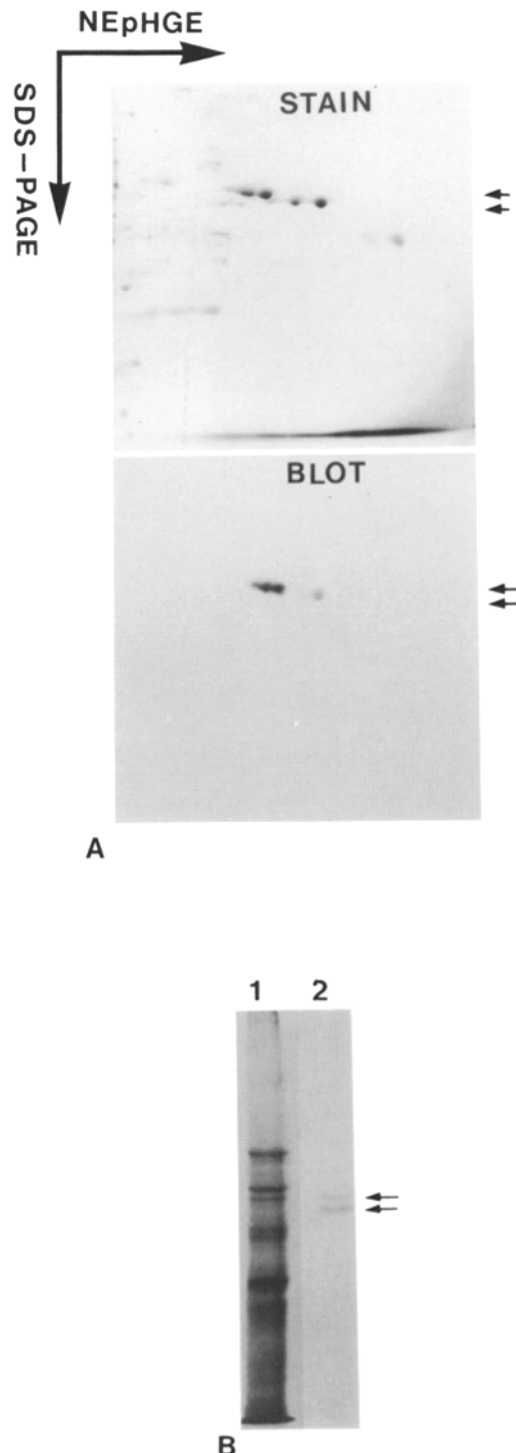

Figure 1. Specificity of antibodies raised against the articulins. (*A*) Two-dimensional (*NEpHGE/SDS-PAGE*) separation of proteins of surface isolates stained with Coomassie blue (CB). In the lower gel the protein separation was similar to *A* but the proteins were transferred to nitrocellulose and probed with the monoclonal antibody 3G1. Isoforms of both articulins (80- and 86-kD, *arrows*) are recognized by this mAb, although the 80-kD isoforms bind less antibody. (*B*) SDS-PAGE of whole *Euglena* cell proteins (CB stained, lane *1*), transferred to nitrocellulose (lane *2*) and incubated with pooled polyclonal (80pp-1 and 86pp-2) antibodies. Arrows indicate the 80- and 86-kD polypeptides specifically recognized by these antibodies. SP2/0 serum (*A*) and preimmune serum (*B*) gave no signal in their respective blots (data not shown).
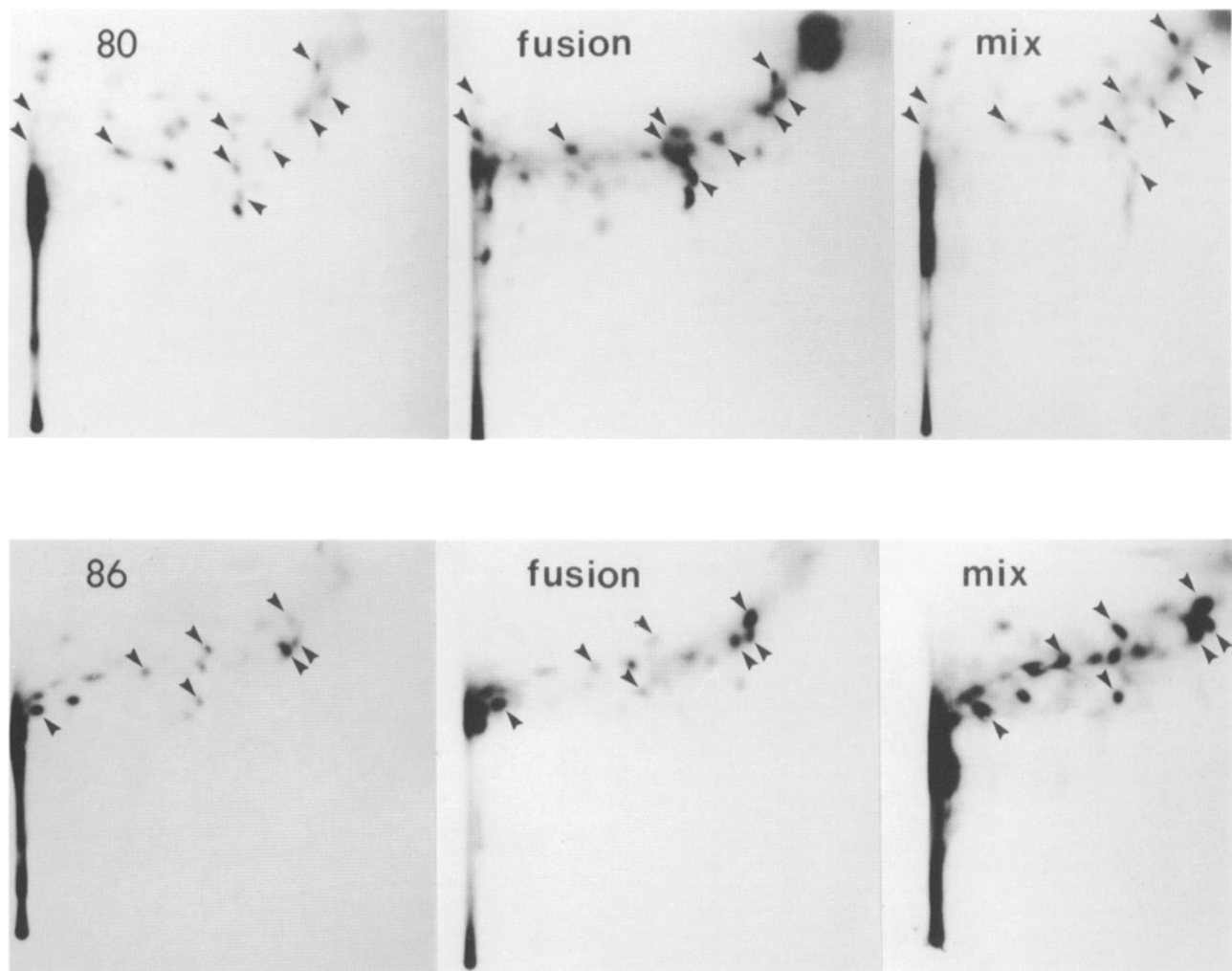
A

Figure 2. cDNA clones encode articulins. (*A*) Peptide maps comparing endogenous articulins (80- and 86-kD) with fusion proteins expressed in λ clone lysogens. Mix is 80-kD articulin + λ80/4 lysogen (*top, right panel*) and 86-kD articulin + λ86/5 lysogen (*bottom right panel*). Arrowheads identify peptides which comigrated in the 80-kD and λ80/4 maps (*top panels*), and in the 86 kD and λ86/5 maps (*lower panels*). (*B*) Immunoblots of proteins expressed in bacteria infected with λ80/4 and with λ86/5 probed with anti-articulin antibodies. Extracts of infected cells induced with IPTG (+ lanes), or untreated (− lanes) were separated by SDS-PAGE, electroblotted to nitrocellulose membranes, and probed with anti-articulin polyclonal antisera (lanes *1-4*) or with mAb 3B2 which is specific for the 80-kD articulin (lanes *5-8*). The position of the induced fusion proteins is shown by arrowheads. (*C*) Antibodies to λ fusion proteins recognize endogenous articulins in immunoblots. Odd numbered lanes, blots of *Euglena* surface isolates (*S*); even numbered lanes, blots of solubilized whole *Euglena* (*E*). (Lanes *1* and *2*) Probes are preimmune serum of anti-λ80/4; (lanes *3* and *4*) anti-λ80/4 fusion protein; (lanes *5* and *6*) preimmune anti-86/5 fusion protein; (lanes *7* and *8*) anti-86/5 fusion protein; (lanes *9* and *10*) mAb 3G1 (anti-articulin).

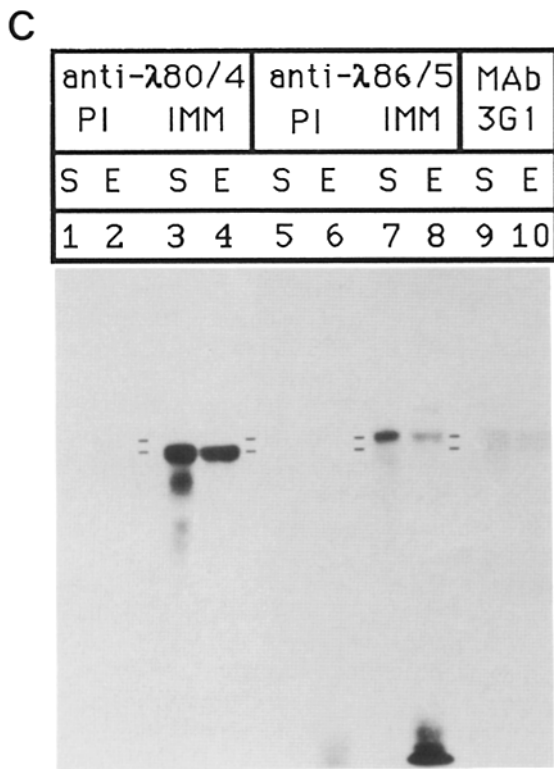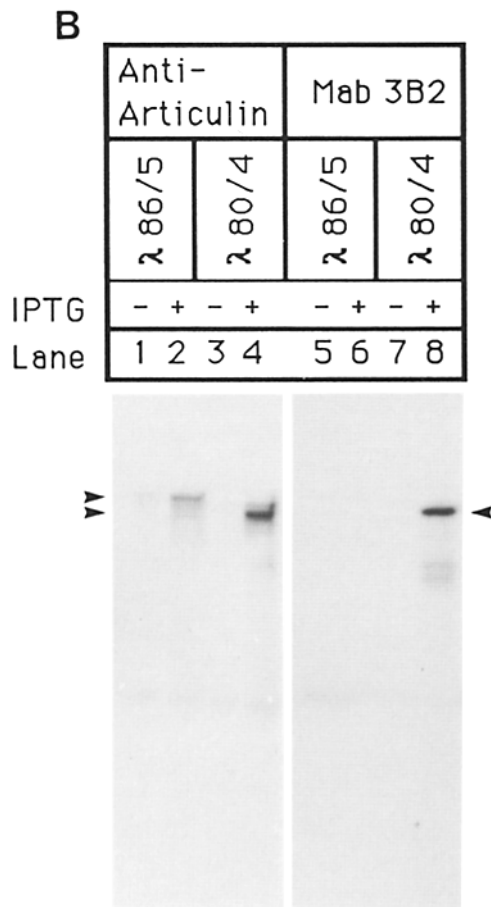separated from one another by eluting with a shallow salt gradient.

The copurification of the articulins through a multistep protocol suggests that they are similar proteins, and this similarity was further evidenced from the results of immunological experiments. Proteins of surface isolates were separated by two-dimensional electrophoresis, transferred to nitrocellulose, and probed with the mAb 3G1. This mAb recognized three isoforms of both articulins labeled (Fig. 1 *A*), although the 86-kD articulin was labeled more intensely. The fact that one mAb bound to both proteins suggests that the articulins are related, that they share at least this one common epitope. Polyclonal antibodies raised against each individual articulin band recognized both articulins in Western

blots, even when the antibodies were affinity purified against their respective proteins (see Materials and Methods). Previous studies using other mAbs which recognized only one or the other protein (Dubreuil and Bouck, 1988) indicated that articulins also have unique domains.

In two-dimensional tryptic maps of the 80-kD articulin, the 86-kD articulin, and a mixture of peptides from both articulins, only a small number of minor peptides appeared to comigrate, but in α-chymotryptic peptide maps there was clear overlap between four peptides (data not shown).

Isolation of cDNA Clones Encoding Both Articulins

Six cDNA clones were recovered from a *Euglena gracilis*

B

	Anti-Articulin		Mab 3B2	
	λ 86/5	λ 80/4	λ 86/5	λ 80/4
IPTG	− +	− +	− +	− +
Lane	1 2	3 4	5 6	7 8

C

anti-λ80/4		anti-λ86/5		MAb 3G1
PI	IMM	PI	IMM	
S E	S E	S E	S E	S E
1 2	3 4	5 6	7 8	9 10

cDNA library (Levasseur, P. J., Q. Meng, and G. B. Bouck, manuscript submitted for publication) after selection with the combined polyclonal antisera (Fig. 1 *B*). These cDNAs could be separated into two groups that under high stringency conditions cross-hybridized with members of the same group but not to members of the other group. Evidence presented below shows that the cDNAs designated 80/4, 80/8, and 80/11 encoded the 80-kD articulin and the cDNAs designated 86/5, 86/6, and 86/7 encoded the 86-kD articulin.

To demonstrate that the cDNA clones encoded articulins, lysogenic strains were generated from the λgt11 clones 80/4 and 86/5 (each the largest member of its cDNA group). The lysogens were induced with IPTG to produce high molecular weight β-gal fusion proteins that were used both for peptide mapping and for generating anti-fusion protein antibodies. (*a*) Peptide maps were constructed for the two authentic articulins and for the two fusion proteins (Fig. 2 *A*; 80, 86, fusions) and for all permutations of paired mixtures. The 80-kD map was most similar to the λ80/4 fusion protein map, and the 86-kD articulin map was most similar to the λ86/5 fusion protein map. Maps of paired mixtures support these conclusions; only the maps of mixtures of similar proteins are shown here (Fig. 2 *A*; 80 + fusion and 86 + fusion). As expected, some peptides do not comigrate since additional peptides are derived from the β-gal portion of the fusion protein. It is also possible that the endogenous *Euglena* articulins may undergo posttranslational modifications that would alter migration relative to the non-modified bacterially expressed fusion proteins.

(*b*) Total proteins from lysogenized bacterial cells were separated by SDS-PAGE, transferred to nitrocellulose, and probed with the anti-articulin antisera used initially to screen the λgt11 library. A high molecular weight protein (presumably β-gal fusion protein) was recognized in both lysogen samples induced with IPTG but not in lysogens not induced with IPTG (Fig. 2 *B*, lanes *1–4*). A similar blot was probed with mAb 3B2 which binds only to the 80-kD articulin (Dubreuil and Bouck, 1988). mAb 3B2 recognized the IPTG induced λ80/4 fusion protein, but not the similarly induced λ86/5 fusion protein (Fig. 2 *B*), thereby providing independent confirmation of the identity of the 80/4 clone.

(*c*) The high molecular weight immuno-positive IPTG-induced proteins expressed in λ80/4 and λ86/5 lysogens were excised from SDS polyacrylamide gels and injected into rabbits. The resulting anti-fusion protein polyclonal antisera were incubated with nitrocellulose blots of *Euglena* polypeptides. Preimmune serum did not bind appreciably to any polypeptides in the *Euglena* samples (Fig. 2 *C*, lanes *1, 2, 5, 6*), whereas the anti-λ80/4 fusion protein antiserum bound strongly to the 80-kD articulin but not to the 86-kD articulin among polypeptides from both whole cell extracts and from surface isolates (Fig. 2 *C*, lanes *3* and *4*). The anti-λ86/5 fusion protein antiserum bound to the 86-kD articulin but not to the 80-kD articulin in both *Euglena* samples (Fig. 2 *C*, lanes *7* and *8*). Independent confirmation of the relative migrations of the articulins was provided by incubating the immunoblots with mAb 3G1 which recognizes both proteins (Fig. 2 *C*, lanes *9* and *10*). Thus, the two antisera to fusion proteins identified the individual, endogenous *Euglena* 80- and 86-kD articulins. No other proteins were recognized that were not also recognized by preimmune serum. There was some apparent degradation of the articulins in the surface
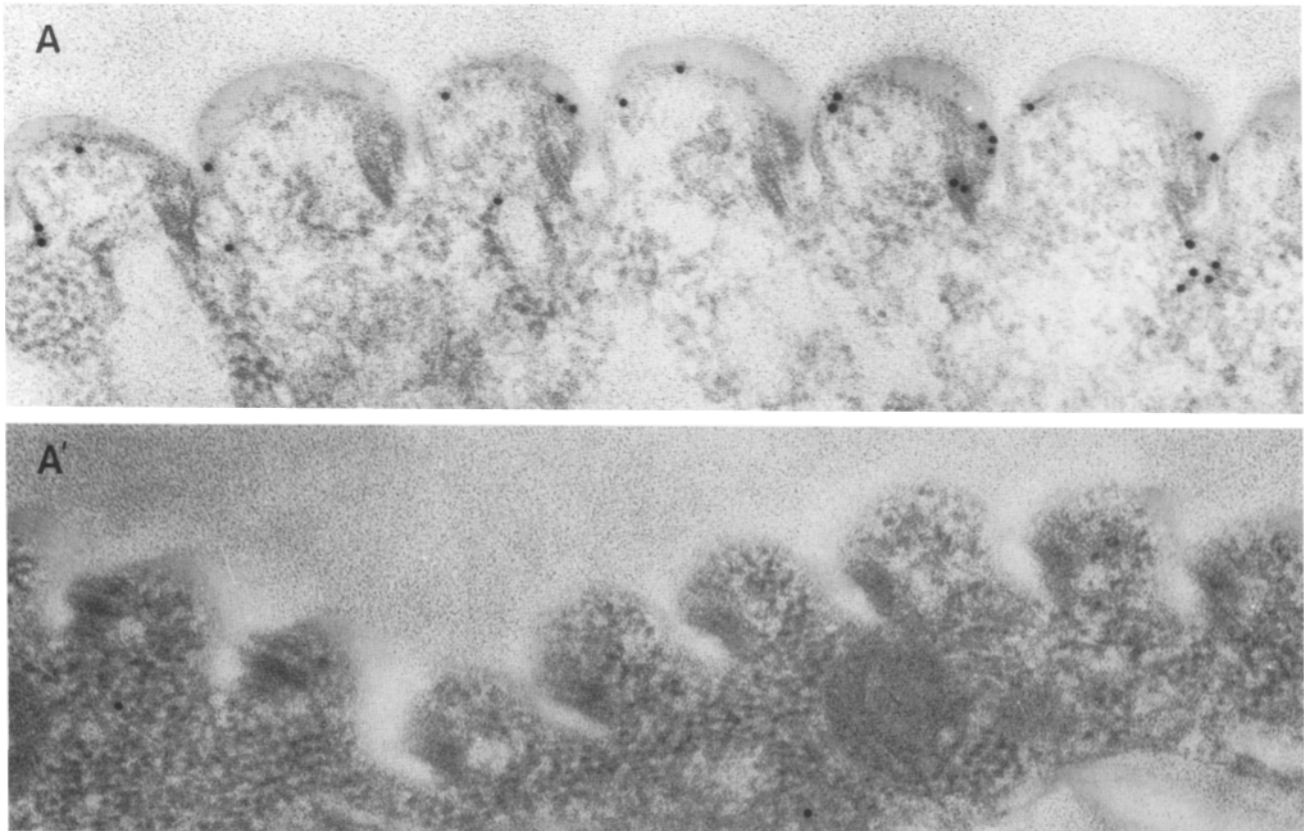
Figure 3. Immunoelectron microscopy localizes fusion protein antibodies in *Euglena*. (*A*) Primary antibody was rabbit anti-80/4 fusion protein. Secondary antibody was 15-nm gold conjugated to goat anti-rabbit. (*A'*) Same as A but primary antibody was preimmune serum. Gold particles are found associated with the plasma membrane that overlies the membrane skeleton. The membrane follows the alternating ridges and grooves that characterize the surface of these cells. Bar, 150 nm.
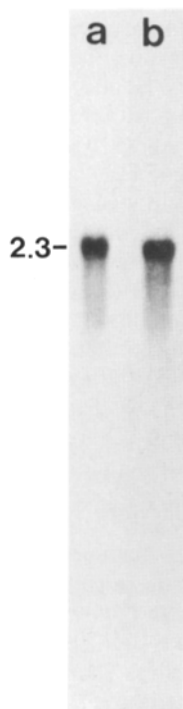

Figure 4. Northern blots probed with articulin encoding cDNAs. Total RNA (15 µg) of *Euglena gracilis* was separated by electrophoresis in 1% agarose under denaturing conditions, transferred to GeneScreen and probed with 80/4 cDNA (lane *a*) and 86/5 cDNA (lane *b*). The 2.3-kb marker was determined from an unrelated mRNA of known size.

isolate sample, but it is clear from the total cell extract lanes that these antisera were virtually monospecific.

(*d*) Antibody raised against the λ80/4 lysogen fusion protein was incubated with thin sections of Lowicryl embedded cells; the 15-nm gold particles of the secondary antibody were associated primarily with the plasma membrane (Fig. 3 *A*) which overlies the 17-nm membrane skeleton (Dubreuil and Bouck, 1985). Over half of the bound gold was localized within the area of surface undulations that represents only 9% of the total cell area (two determinations), i.e., a six-fold greater concentration of immuno-binding in the peripheral region than in other parts of the cell. Control sections (Fig. 3 *A'*) incubated with preimmune serum showed ∼90% fewer gold particles with no significant concentration at the cell surface (four determinations).

To determine whether the isolated cDNAs represented the full coding sequence Northern blots of total RNA from *Euglena gracilis* were probed with cDNAs 80/4 and 86/5. Both cDNAs hybridized with RNA of ∼2.3 kb (Fig. 4). Since this is about the length of the cDNAs, it seemed likely that these were nearly full-length copies of their respective mRNAs. Primer extension (data not shown) suggested that these cDNAs were lacking only 10 (80/4 cDNA) and 20 (86/5 cDNA) bp of their characteristic *trans*-spliced 5' ends (see Discussion).

80 kD

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M S Y L A G A P V T A A A S Y P V V P G A A A Y P V G A S Y P A V
A Q A S V L R A N A Q F A G Q R V I R K E V V G N V E Y I P V Q H
N H E V E L I E R E F I V P V E K I V Q R H V P V P V E R I V Q R
R V P V P R Q V P V P Q R V E I P V P V E R I Q H R Q V P Y P V E
Q I V E K R I P V P V T Q I V E Q A V E V P V P V H R R V I Q Q V
P V P H A V V R E V I R H E P Y P V T K E V I R Q V P V E V P R E
V V R Q V T V D V P V Q V P Q H V Q V P Y P V E K V V H R Q V P Y
P V E K V V Q R Q V P Y P V Q K I V E R Q V Q V P Y E V L V P E R
V E I P V P H E V I T H R D V P V P Q E V I R T V Q V P V P V E Q
I V H R D V P Y P V E Q I V E K V V Q V T R Q V T V P E I V Q V P
V P H E V I V E R R V P V P V E R I I H K A V P Y P V E K Q I V E K
I V Q V P V V P Q Y Q K V P V Q V P V V P V E R I V T R D V P Y P V E
Q I V D K V V E R Q V P V V P T P V Q V P V P T P V Q V P Y P V E K
I V D R P V P H E V V R V V E R R V E V P Y D V P V P V I E T V Q
V P H E V I R T V E V P F P V E Q I V E K I V E K I V H V P V P V
T R Q E H V T R Q V V Q N T L Q Q T R A P P Q Q L G T Q V L P G R
D L G V T S G I V R G G P A A Y G A Y G A T P V A A A P V T Y G S
V A A A P V T Y G A A A P V T Y G A A P V T A Y G A P P V Y A G A P
A V V T A P S S V T R Y G Y G P P R P A Y P A T V P V A Y A A A P
T E L R N D D F D W P P V P S V C G V Q R Q P Q
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86 kD

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M S W V A A Q T Q Q G A F A L D A A D G R I D G K Y F G S N V G
V S V P G A P V T Y G A A A P V T S Y A A P A A V T S F A A T A A
P V T S F G A Y G A Y A P A C P P C A T G P R V V N D P L E T R F
V D V V K Q V E T I R S V D V P V P H E V V R T V D V P E H Y D V
P V P H A V H V Q V P Y P V D K F V D V P V P H T I Q K I V E T R
V P Y P V Q Q V V Q R R V E R P Y D V P V V E R V N V P Y P V E Q
V V E R R V P V P V E Q I V E R V V Q V P V P R L V E K V V Q V H
R Q V P V P V R V E V P V P R E V I R T V D V P V P R E V V R T Q
D V P V P V E Q I V E K V V Q V P V P V Q K K V I Q H V Q V P Y P
V Q K I V D R P V P Y P V E K I V E Q K V P Y A V Q K V I D R P V
P E P V Q K I V E R R V D V P V P V E V K V R Q E V R V P Y P V Q K I
V D R P E P Y P V D K V V E V P Q P Y P V Q K V V E R R V E V P H
V I Q V R E E V R V P Y T V D K V V D R P V P Y P V T K E V V R Y
V D R P V P Q P Y E V R V P Q P Y E V K V P V E Q I R Y R D V P V
P V E R I V E K V V Q V P V P R Q V P V K Q I Q Q V P V P V E K I
V H V Q R P Y P V Q K V V V R E V V K H V Q V P H E I I Q R V E H
V Q H V V Q P V E V I E E P V I Q Q I V T I N K R T I Q G Q P Y V
A N T T T N V I G Q E V R G A Y A A G A Y G A T V T G P A V A T G
Y G Q S V T G Y G Q S V T G Y G Y G A A A P V A V A G S Q G G A L
S L D L A D G R L D G R F Y G A P V V S A S P F
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77-	F I V P V E K I V Q R H	-88			113-	V P V P H E V V R T V D	-124
89-	V P V P V E R I V Q R R	-100			125-	V P E H Y D V P V P H A	-136
101-	V P V P R Q V P V P Q R	-112			139-	V Q V P Y P V D K F V D	-150
115-	I P V P V E R I Q H R Q	-126			151-	V P V P H T I Q K I V E	-162
127-	V P Y P V E Q I V E K R	-138			163-	T R V P Y P V Q Q V V Q	-174
139-	I P V P V T Q I V E Q A	-150			177-	V E R P Y D V P V V E R	-188
153-	V P V P V H R R V I Q Q	-164			189-	V N V P Y P V E Q V V E	-200
165-	V P V P H A V V R E V I	-176			203-	V P V P V E Q I V E R V	-213
179-	E P Y P V T K E V I R Q	-190			215-	V Q V P V E R L V E K V	-226
191-	V P V E V P R E V V R Q	-202			227-	V Q V H R Q V P V P V R	-238
203-	V T V D V P V Q V P Q H	-214			241-	V P V P H E V I R T V D	-252
215-	V Q V P Y P V E K V V H	-226			253-	V P V P H E V V R T Q D	-264
229-	V P Y P V E K V V Q R Q	-240			265-	V P V P V E Q I V E K V	-276
241-	V P Y P V Q K I V E R Q	-252			279-	V P V P V Q K K V I Q H	-290
253-	V Q V P Y E V L V P E R	-264			291-	V Q V P Y P V Q K I V D	-302
267-	I P V P H E V I T H R D	-278			303-	R P V P Y P V E K I V E	-314
279-	V P V P Q E V I R T V Q	-290			315-	Q K V P Y A V Q K V I D	-326
291-	V P V P V E Q I V H R D	-302			327-	R P V P Y P V Q K I V E	-338
303-	V P Y P V E Q I V E K V	-314			341-	V D V P V E V K V R Q E	-352
315-	V Q V T R Q V T V P E I	-326			353-	V R V P Y P V Q K I V D	-364
327-	V Q V P V P H E V I V E	-338			365-	R P E P Y P V D K V V E	-376
341-	V P V P V E R I I H K A	-352			377-	V P Q P Y P V Q K V V E	-388
353-	V P Y P V E Q I V E K I	-364			391-	V E V P H V I Q V R E E	-402
367-	V P V P Q Y Q K V P V Q	-378			403-	V R V P Y T V D K V V D	-414
379-	V P V P V E R I V T R D	-390			415-	R P V P Y P V T K E V V	-426
391-	V P Y P V E Q I V D K V	-402			429-	V D R P V P Q P Y E V R	-440
407-	V P V P T P V Q V P V P	-418			441-	V P Q P Y E V K V P V E	-452
421-	V Q V P Y P V E K I V D	-432			459-	V P V P V E R I V E K V	-470
433-	R P V P H E V V R V V E	-444			473-	V P V P R Q V P V K Q I	-484
447-	V E V P Y D V P V P V I	-458			487-	V P V P V E K I V H V Q	-498
461-	V Q V P H E V I R T V E	-472			499-	R P Y P V Q K V V V R E	-510
473-	V P F P V E Q I V E K I	-484			515-	V Q V P H E I I Q R V E	-526
491-	V P V P V T R Q E H V T	-502			529-	Q H V V Q P V E V I E E	-540

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V P V P V - - - V - - -            V P V P - - V - - - V -
        e v i     v                        y p/c   v/k   e
```

Figure 5. Deduced amino acid sequences of articulins from cDNA clones. Shaded areas represent the 12-amino acid repeats of the core domains. Underlined amino acids are 7-amino acid repeats at carboxy domain of the 80-kD articulin and of the amino domain of the 86-kD articulin. Charged amino acids are indicated by (+) and (−) over the single letter code. The repeats of the core domain are aligned in the bottom panel. Numbers refer to the position of that amino acid relative to the start methionine. Amino acids of the consensus sequence (*upper case*) were present in > 1/2 of the repeats; lower case letters were amino acids present in > 1/3 of the repeats. Both nucleotide and amino acid sequences are available from EMBL/GenBank/DDBJ under the accession numbers Z13962 and Z13963.
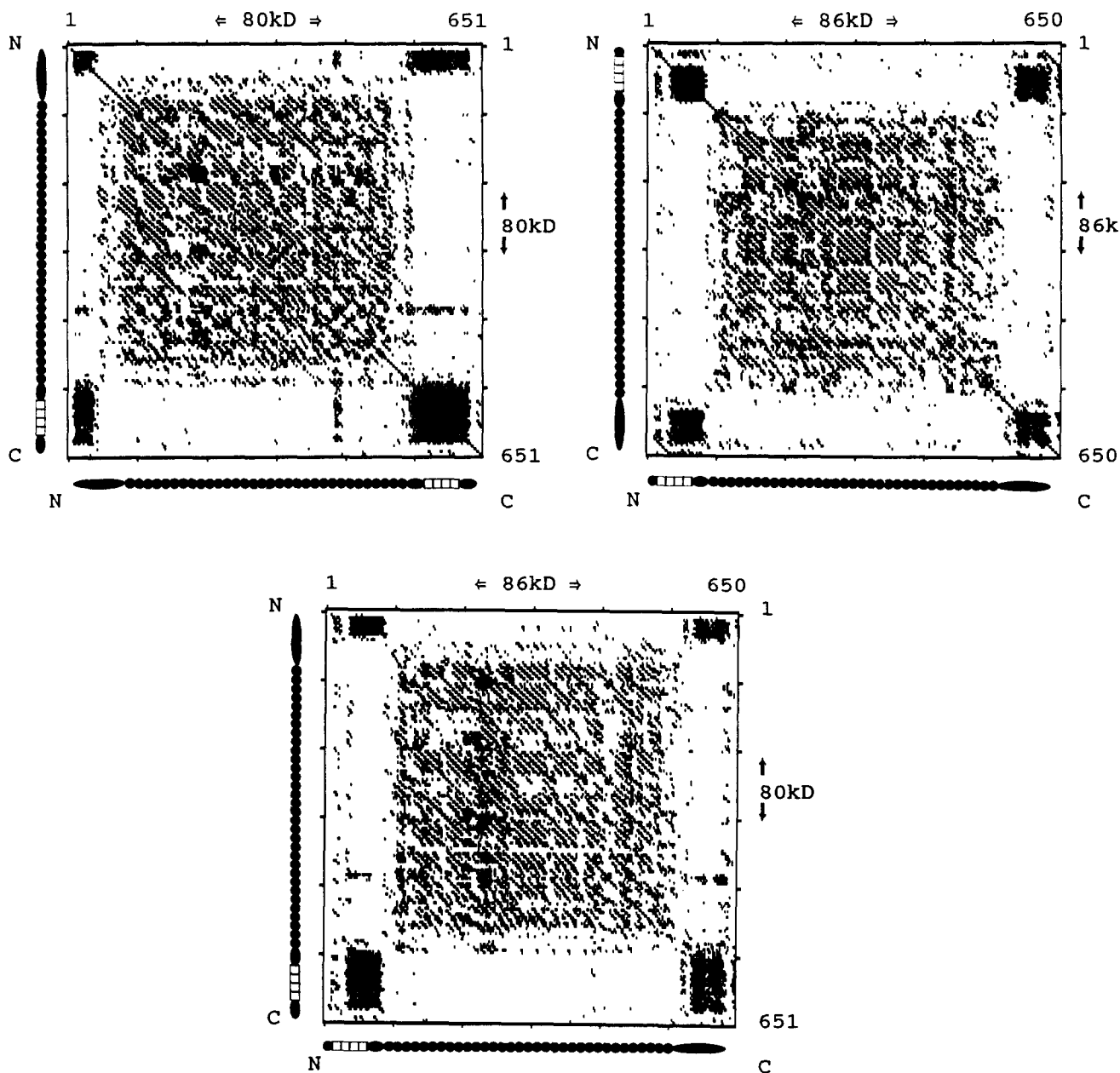
Figure 6. Homology plots of articulin amino acids. Each dot is a match of seven amino acids over a stretch of 12 amino acids (check size) plotted with PROSIS (Hitachi). (*A*) Homology plot comparing 80-kD amino acids with 80-kD amino acids. The central core domain is divided by gaps into a gridwork of ~64 more or less distinct subregions which are about three- to fourfold larger than the 12 amino acid repeats. The strong homology profiles at the COOH terminus are the seven-amino acid repeats. (*B*) Homology plot comparing 86-kD amino acids with 86-kD amino acids. Profiles are similar to that of *A* in that the core domain shows a repetitive core domain separated by gaps into subregions of unequal size. 7 amino acid repeats in the NH₂ terminus are seen as a strong homology area in upper left corner. (*C*) Homology plot comparing 80-kD amino acids with 86-kD amino acids. The central core of 12-amino acid repeats shows strong homology, and the larger subregions as defined by gaps persist. In the schematic diagrams of the articulins shown along the plot axes, the circles represent the 12-amino acid repeats of the core domains, and the white boxes are the 7-amino acid repeats.

The Two Articulin Sequences Are Similar

From the complete nucleotide sequence for cDNA 80/4 (2,221 bp) it was evident that cDNA 80/4 encoded only one large open reading frame (ORF) that predicted a 651-amino acid protein with a molecular weight of 72,111 D (Fig. 5). cDNA 86/5 (2,115 bp) also yielded only one large ORF that would encode a 650-amino acid protein of 71,897 D (Fig. 5). These predicted molecular weights are considerably less than previous determinations made from SDS polyacryl-

amide gels (i.e., 80 and 86 kD), but there was only a single methionine residue within each ORF; hence there were no other potential translation initiation sites. Moreover, it is unlikely that the 5' sequences extend much beyond those present in the cDNA clone (see above; also Discussion). Overlapping sequence data from the 80/4, 80/8, and 80/11 cDNA clones extended the 3' untranslated nucleotides to a long stretch of As, presumably the poly(A) tail of the corresponding mRNA.

Both proteins had an unusually large number of valine

and proline residues (80/4: valine = 25%, proline = 15%; 86/5: valine = 26%, proline = 13%) located predominantly within a central "core domain." The latter consists of 33 ± tandem repeats (Fig. 5, *shaded areas*) with occasional insertions, and with many charged residues. Within the repeats valine is present in almost every second position as indicated in the consensus at the bottom of the aligned sequences (Fig. 5). Homology plots (PROSIS software, Hitachi, San Bruno, CA) displayed graphic similarities, particularly in the core domain. When the sequences were compared to themselves or with each other, subdomains of nonuniform sizes became evident (bordered by gaps that appear as a gridwork in the core domains Fig. 6). The overall prediction of secondary structure was an extended β-sheet, and margins of the subdomains correlated frequently with predicted turns in the β-sheets.

The two articulin amino acid sequences overall were 47% similar and had 37% identity; within the core domains there was 58% similarity and 50% identity. Outside the core domain there was less similarity but four copies of a hydrophobic seven-amino acid repeat (consensus: APVTYGA) were present at the carboxy terminus of the 80-kD articulin and at the amino terminus of the 86-kD articulin (Fig. 5, *underlined sequences*).

Articulin homologues have been reported in other euglenoids (Bricheux and Brugerolle, 1987) and in a dinoflagellate and a ciliate (Vigues et al., 1987) using polyclonal antibodies. Our antibodies bound no proteins from human RBC, *Drosophila* or *Tetrahymena* in Western blots, and the articulins bound no human erythrocyte proteins in ligand binding assays on nitrocellulose filters. Preliminary immunoblotting experiments with the anti-80-kD fusion protein showed binding to polypeptides from *Trypanosoma brucei*, but the anti-86-kD fusion proteins antibody did not bind (our unpublished results). No significant similarity was found between the articulin cDNAs or their predicted proteins and those cataloged in the EMBL, GenBank, NBRF-PIR, and SWISS-PROT databanks. These data suggest that the euglenoids (and perhaps the closely related trypanosomids) have evolved a novel set of membrane skeletal proteins for maintaining cell form and membrane integrity.

Discussion

The Articulins Are Related Proteins with Similar Core Domains

The two major proteins (articulins) of the *Euglena* membrane skeleton were reported to be basic phosphoproteins with similar M_rs of 80 and 86 kD (Bricheux and Brugerolle, 1987; Dubreuil et al., 1992). The articulins also copurified through a number of different separation methods (Dubreuil and Bouck, 1985). In the present report we show that both affinity-purified polyclonal antibodies and at least one monoclonal antibody bind to both articulins, therefore, suggesting that the two proteins share common epitopes. Direct evidence from the predicted amino acid sequences solidifies these findings, as the proteins encoded by articulin cDNAs have 47% overall similarity; both proteins have a conserved core domain that is 58% similar and contain 33 12-amino acid tandem repeats with similar consensus sequences. In the more divergent NH₂- and COOH-terminal

domains there is a second set of seven-amino repeats at opposite ends of the two proteins. Yet the cDNA sequences are distinctly different and thus it seems likely that the 80- and 86-kD articulins are encoded by separate genes.

That the two sets of recovered cDNAs encoded articulins was demonstrated first by comparing peptide maps of authentic articulins with those of cDNA encoded fusion proteins, second by immunoblots using antibodies against fusion proteins and authentic articulins, and third by immunolocalization of fusion protein antibodies at the peripheral membrane skeleton. Moreover, the general properties predicted from the amino acid sequence (e.g., basic isoelectric point, predominance of hydrophobic amino acids) are consistent with the general properties of the articulins, although the predicted molecular masses (~72 kD) are considerably less than that estimated from migration in acrylamide gels (80 and 86 kD). The cDNAs were nearly full length as judged from Northern blots, but efforts to confirm this by primer extension were not completely successful as extension was terminated ~20 nucleotides 5' to the putative start site possibly due to the secondary structure or the presence of a poly-uridine tract (consensus of eight thymidines in the cDNAs) in the *trans*-spliced 5' leader. Sequences corresponding to a portion of the *trans*-spliced leader RNA, found on the terminus of all or most *Euglena gracilis* mRNAs (Tessier et al., 1991) were identified in the 5' termini of both articulin cDNAs (Table I) 10 bp upstream of the putative start codons. It seems likely therefore that the methionines are the actual start sites for the articulins, and the differences in predicted relative to observed molecular weights is the result of anomalous migration of these proteins in SDS polyacrylamide gels.

Both articulins are characterized by internal tandem repeats, a feature common to many membrane skeletal proteins including the spectrins (reviewed in Bennett, 1990). It is the 106-amino acid repeat arranged in a triple α-helical conformation that gives spectrin a flexible rod-shaped structure, and in turn gives strength and elasticity to the erythrocyte membrane. Both the spectrin superfamily and the filamin family of actin-binding proteins contain central domains with tandem repeats. The tandem repeats function in binding accessory proteins and in dimer formation, but the central domains also separate the NH₂- and COOH-terminal regions that bind actin and function in self-association (for review see Hartwig and Kwiatkowski, 1991). The 12-amino acid repeats of the *Euglena* articulins are predicted to form predominantly extended β-sheets. An extended conformation would be consistent with the filaments that reconstituted perpendicularly to the plasma membrane from fractions highly enriched in articulins (Dubreuil and Bouck, 1988), but what role the NH₂-terminal, COOH-terminal, and central domains play in self-assembly and in binding to the integral membrane protein IP39 (Rosiere et al., 1990) remains to be determined.

From earlier biochemical studies it was evident that the articulins were basic, hydrophobic proteins, and in general the predicted protein sequences are consistent with these findings. The predicted pIs for the 80- and 86-kD articulins were 8.32 and 8.45, respectively, and on NEpHGE gels the articulins extracted from the membrane skeleton migrate as basic proteins (Fig. 1; also, Bricheux and Brugerolle, 1987). Articulins bound to phenyl Sepharose columns under high

Table I. 5' Leader Sequences of Euglena cDNAs

5'		3'
86ART	--TTCG	CCGAACAACA(ATG)*
80ART	---TTTTTTTTCG	CGTCGGACAT(ATG)*
HMBS	---TTTTTTTTCG	GAAG(ATG)‡
α-TUB	---TTTTTTTTTCGCTCAAAACTCTTTCAAA(ATG)§	
β-TUB	--------------------------------------AAAGTGTCTATTTTTTTTCG	ATCACAA(ATG)‖
TEF-1α	----------------------------------TTTCTGAGTGTCTATTTTTTTTCGCAAT(ATG)¶	
SSU	--TTTTTTTTTTCGCACTCTTGCCGGCTCTCATTACG(ATG)**	
LS	------------------------------TTTCTGAGTGTCTATTTTTTTTCG – consensus‡‡	

The 5' ends of various sequenced *Euglena* cDNAs are aligned for comparison with the authentic *trans*-spliced leader sequences (LS) of Tessier et al. (1991). Shaded regions conformed to the consensus. (ATG) encodes the presumptive start methionine for each protein. The articulin (80ART and 86 ART) cDNAs have incomplete 5' termini that are in agreement with the leader consensus. * This paper; (‡) Sharif et al., 1989; (§) Levasseur, P. J., Q. Meng, and G. B. Bouck, manuscript submitted for publication; (‖) Schantz and Schantz, 1989; (¶) Montandon and Stutz, 1990; (**) Chan et al., 1990; (‡‡) Tessier et al., 1991.

salt conditions, could be eluted with reduced salt and have low solubility in aqueous solutions — all properties of hydrophobic proteins. From the protein sequence data, it is evident that both articulins are enriched in the hydrophobic (aliphatic) amino acid valine (25 and 26% molar ratios for the 80- and 86-kD proteins), particularly in the core domain where nearly every second residue is valine. Somewhat surprisingly, the overall hydropathy index predicted for both the entire proteins and the core domains was slightly negative (hydrophilic), suggesting that strongly hydrophobic sequences such as the seven-amino acid repeats at the carboxy terminus of the 80-kD articulin and at the amino terminus of the 86-kD articulin may be exposed on the outer surface of the folded protein. These short hydrophobic repeats are of additional significance in that they are positioned for possible head-to-tail hydrophobic interactions between the articulins, an association postulated from earlier reassembly studies (Dubreuil and Bouck, 1988).

The Articulins May Have Evolved from a Common Ancestral Protein

The two articulins were 47% similar in amino acid content. They had the same organization into amino-, carboxy-terminal and core domains, and they were about the same size (650 and 651 amino acids). Thus it seems likely that an ancestral articulin-like gene has undergone gene duplication, followed by divergence of two new genes that encode the 80- and 86-kD articulins of *Euglena gracilis*. Since there are pairs of putative articulin homologues found in the surface complexes of many different euglenoids (Bricheux and Brugerolle, 1987), the duplication of the ancestral articulin gene must have occurred early in (or prior to) the evolution of this group. Many gene families are thought to have evolved by a similar mechanism, including the spectrin gene family, i.e., the spectrins, α-actinin and dystrophin (Speicher and Marchesi, 1984; Baron et al., 1987; Wasenius et al., 1985; Koenig et al., 1988). Sequence and structural comparisons of the articulin genes from various euglenoid genera will be of considerable interest in following the divergence that seems to produce the different patterns of membrane skeletons (on a common theme) within this group and in identifying conserved functional domains in this novel class of membrane cytoskeletal proteins.

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