A Candidate Molecule for the Matrix Assembly Receptor to the N-terminal 29-kDa Fragment of Fibronectin in Chick Myoblasts*

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Myoblast surface proteins with binding activity toward the N-terminal 29-kDa fragment of fibronectin were identified by two different experimental techniques: one involves radiolodination of the cell surface proteins, followed by solubilization with Triton X-100 and affinity purification on a Sepharose column conjugated with the 29-kDa fragment, and the other involves cross-linking of the 29-kDa fragment to the cells metabolically labeled with [35S]methionine, followed by immunoprecipitation with anti-29-kDa IgG. Both approaches revealed that primary cultures of chick myoblasts contain the 66- and 48-kDa proteins that bind to the 29-kDa fragment. These binding proteins were then purified to apparent homogeneity by two successive chromatographies of the solubilized extracts of 12-day-old embryonic muscle on wheat germ agglutinin-agarose and 29-kDa fragment-Sepharose columns. However, the 48-kDa protein was found to be derivated from contaminating fibroblasts upon immunoblot analysis of the myogenic cell lines, rat L9E63 and mouse C2A5, and cultured fibroblasts using the antibody raised against the 66-kDa protein.

Anti-66-kDa IgG inhibited the binding of the 125-I-29-kDa protein to the primary culture of myoblasts in a dose-dependent manner. On the other hand, the same antibody showed little or no effect on the initial binding of 125I-fibronectin to the cell surface, but dramatically inhibited its incorporation into deoxycholate-insoluble matrices. Furthermore, Fab fragments of anti-66-kDa IgG completely blocked the incorporation of fluoresceinated fibronectin into matrices but not its binding to the cell surface. These results suggest that fibronectin matrix assembly is mediated at least in part by the interaction of the 66-kDa protein with the N-terminal type I domain of fibronectin.

Fibronectin binds to a wide variety of cells, such as fibroblasts, HT-1080 cells, and endothelial cells, and assembled into extracellular matrices that are essential for cell attachment, migration, differentiation, and organ morphogenesis (1-3). Many of these cellular processes have been attributed to the binding of fibronectin with integrin on the cell surface through the Arg-Gly-Asp (RGD)-containing cell-adhesive site in fibronectin molecule. And the binding of fibronectin to cell surface receptors of the integrin family has well been documented (4-6). In addition to the RGD site, fibronectin has been shown to contain a distinct cell-binding site that also appears essential for matrix assembly. The second site is located within the N-terminal 29-kDa fragment of fibronectin, which contains the first five repeats of the type I homologous domain (7-11). Recently, a 67-kDa protein that binds specifically to the 29-kDa fragment of fibronectin has been purified from the insoluble fractions of human U937 cells and rat peritoneal macrophages (12). This protein may represent a unique macrophage surface binding protein for the N terminus of fibronectin, and yet is unlikely to function in matrix assembly because macrophages do not produce matrices. A few other proteins have been reported to interact with the N-terminal domain of fibronectin. McDonald and co-workers (13) have identified a 150-kDa protein complex by cross-linking the 29-kDa fragment to the surface of fibroblasts. An additional 18-kDa fibroblast protein with N-terminal fibronectin binding activity has also been identified (14). However, the latter two proteins have not yet been purified.

A prominent event in the differentiation of skeletal muscle cells is the fusion of mononucleated myoblasts into multinucleated myotubes. This process accompanies various cellular events, such as a direct physical interaction of plasma membrane (15), reorganization of cytoskeletons (16, 17) and extracellular matrices (18). A number of reports have suggested that fibronectin, a cell surface glycoprotein, exerts a profound effect on myoblast fusion (for a review, see Ref. 19). For example, treatments of exogenous fibronectin to rat myoblast cultures have been shown to block the membrane fusion (20). It has also been demonstrated that the treatment of antibodies directed to integrin inhibits the fusion process (21). In addition, we have recently demonstrated that the level of fibronectin in chick embryonic myoblasts decreases during the course of myogenic differentiation, and this decrease is closely correlated with the fall in the extent of binding of the 29-kDa fibronectin fragment to the cell surface (22).

In an attempt to elucidate the role of fibronectin and the surface binding of its 29-kDa fragment in the regulation of myogenic differentiation of cultured myoblasts, we have purified a protein of 66 kDa that specifically binds to the N-terminal fragment of fibronectin from the insoluble fractions of the cells. In addition, we provide several lines of evidence that the 66-kDa protein is a potential candidate for fibronectin matrix assembly receptor.

EXPERIMENTAL PROCEDURES

Materials—Fibronectin and its N-terminal 29-kDa fragment were purified as described (22, 23). The proteins were radiolabeled with}

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1 The abbreviations used are: RGD, Arg-Gly-Asp; FITC, fluorescein isothiocyanate; TRITC, tetramethyl rhodamine isothiocyanate; MEM, Eagle's essential minimal medium; DMEM, Dulbecco's modified Eagle's medium; NEM, N-ethylmaleimide; DSP, dithiobis(succinimidyl) propionate; cpm, counts/min.
Na\[^{35}\text{S} \] using Iodo-Beads (Pierce). Antibody against the 29-kDa fragment was prepared by injecting the peptide into albino rabbits. To prepare antibody against the 29-kDa fragment-binding protein (i.e., the 66-kDa protein; see below), proteins eluted from a Sepharose column conjugated with the 29-kDa fragment were electrophoresed on 10% (w/v) polyacrylamide slab gels in the presence of sodium dodecyl sulfate (SDS) (24). After staining the gel with Coomassie R-250, the protein bands corresponding to 66 kDa were cut out, minced, and injected. IgGs were purified by protein A-Sepharose column chromatography (25). Fab fragments of the anti-66-kDa IgGs were prepared as described (26). Anti-\(\beta\), integrin antisera was kindly provided by Dr. A. F. Horwitz (University of Illinois).

The 29-kDa fragment-affinity column was prepared by coupling the purified 29-kDa polypeptides to CNBr-activated Sepharose (27). Approximately 5 mg/ml of the peptides were covalently attached to the column. Fluorescein-labeled fibronectins were prepared by incubating 10 \(\mu\)g/ml of fluorescein isothiocyanate (FITC) and 2 mg/ml of fibronectin in 4 mM NaHCO\(_3\)/NaHCO\(_3\), pH 9.0, containing 0.15 \(\mu\)M NaCl. After incubating the mixture overnight at 4°C, the FITC-labeled proteins were further purified by chromatography on a Sephadex G-25 column equilibrated with phosphate-buffered saline.

Cell Cultures—Myoblasts from breast muscle of 12-day-old chick embryos were prepared as described (28). The cells were plated at a density of \(5 \times 10^5\) cells/ml in Eagle's minimal essential medium (MEM) containing 10% (v/v) horse serum, 10% (v/v) chick embryo extract. The cultures were further purified by chromatography on a Sephadex G-25 column equilibrated with phosphate-buffered saline.

Cell Surfaces—Myoblasts from breast muscle of 12-day-old chick embryos were prepared as described (29). C2A3 myoblasts, subcloned from the C2 line of mouse satellite cells (29), were grown in DMEM containing 10% horse serum as described (28). C2A3 myoblasts, subcloned from the C2 line of mouse satellite cells (29), were grown in DMEM containing 10% fetal bovine serum. Cultures of skeletal muscle fibroblasts and skin fibroblasts were obtained from 12-day-old chick embryos as described (30, 31). The cells were cultured in DMEM containing 5% fetal bovine serum. The cells used for experiments were between passage 3 and 5. All cells were cultured in a humidified incubator in an atmosphere of 95% air and 5% CO\(_2\) at 37°C.

Radiolabeling of Cell Surface Proteins—Myoblasts were cultured for 24 h and detached from the plates by treating with 5 mM EDTA for 15 min. After centrifugation, the pellet was resuspended in phosphate-buffered saline containing 2 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, and 2 mM EDTA. The cells were surface labeled for 3 min with 1 nCi of Na\[^{35}\text{S} \]methionine.

Immunoblotting Analysis of the 29-kDa Fragment-Binding Proteins—In order to immunoprecipitate the 29-kDa fragment-binding proteins, chick myoblasts were cultured for 30 h with 200 \(\mu\)g/ml of FITC-labeled fibronectin; the cells were then washed four times with ice-cold Eagle's balanced salt solution, and the extracts were prepared by resuspending sequentially in 20 mM Tris-HCl, pH 8.3, containing 1% (w/v) deoxycholate 2 mM phenylmethylsulfonyl fluoride and 2 mM NEM and the Tris buffer containing 4% SDS (7). In certain cases, the cells were solubilized only with 1% SDS. The radioactivity in the extracts was determined using a gamma counter.

Fluorescence Microscopy—Chick myoblasts were grown for 30 h on coverslips and rinsed twice with Eagle's balanced salt solution. When assayed for the incorporation of exogenous fibronectin into matrices, the cultures were incubated for 3 h with 200 \(\mu\)g/ml of FITC-labeled fibronectin, rinsed with DMEM, and further incubated in DMEM containing 10% horse serum for the next 24 h. For determination of the binding of fibronectin to cell surface, incubations with FITC-labeled fibronectin were performed only for 15 min. After the incubations, the cells were fixed with 3.5% (v/v) paraformaldehyde for 30 min, mounted with glycerol, and observed under a fluorescence microscope (Nikon). When assayed for the ability of the cells to themselves by making fibronectin matrices, the cultures were incubated in DMEM containing 10% horse serum as above but in the absence of FITC-labeled fibronectin. After the incubation, they were fixed and treated with anti-fibronectin rabbit IgG and then with goat anti-rabbit IgG conjugated with FITC at room temperature for 1 h.

To determine whether the 66-kDa protein co-localizes with the site of fibronectin matrix formation, chick myoblasts cultured for 30 h were incubated for 30 min with FITC-labeled fibronectin. The cells were fixed and treated with the 66-kDa IgG and then with anti-rabbit IgG conjugated with tetramethyl rhodamine isothiocyanate (TRITC) (Sigma) at room temperature for 1 h. The double-labeled samples were observed as above.

RESULTS

Identification of the 29-kDa Fragment-Binding Proteins—To identify the 29-kDa fragment-binding proteins, chick myoblasts were radioiodinated using lactoperoxidase and solubilized with 1% Triton X-100. The solubilized proteins were loaded onto a Sepharose column conjugated with the N-terminal 29-kDa fragment of fibronectin, competitively eluted with soluble 29-kDa fragments, electrophoresed in the presence of SDS, and autoradiographed. Fig. 1A shows that two distinct proteins are capable of binding to the 29-kDa fragment. These proteins have apparent molecular masses of 66 and 48 kDa under both the reducing and nonreducing conditions. Therefore, it is unlikely that these polypeptides are covalently linked with each other.

To clarify further the presence of the 29-kDa fragment-binding proteins on the surface of chick myoblasts, the cells that had been metabolically labeled with \(^{35}\)S-methionine were cross-linked to the 29-kDa fragment using dithiobis(succinimidyl propionate) and solubilized with 1% Triton X-100. The solubilized proteins were immunoprecipitated using anti-29-kDa IgG and protein A-Sepharose. Precipitates were resus-
The 29-kDa Fragment-binding Proteins—In order to isolate the 29-kDa fragment-binding proteins, membrane proteins were obtained from breast muscle tissues of 12-day-old chick embryos and solubilized with 1% Triton X-100. The solubilized proteins were chromatographed on a wheat germ agglutinin-agarose column as described under "Experimental Procedures." The purified proteins were cross-linked with the 29-kDa fragment, solubilized by 1% Triton X-100, and immunoprecipitated using anti-29-kDa antiserum as described in the text. The precipitates were subjected to electrophoretic analysis as above but only under reducing conditions.

Purification of the 29-kDa Fragment-binding Proteins—In order to isolate the 29-kDa fragment-binding proteins, membrane proteins were obtained from breast muscle tissues of 12-day-old chick embryos and solubilized with 1% Triton X-100. The solubilized proteins were chromatographed on a wheat germ agglutinin-agarose column. Proteins bound to the column were eluted with N-acetylglucosamine and electrophoresed in duplicate on 10% slab gels in the presence of SDS. A. one of the gels was stained with Coomassie R-250, and, B, the other was subjected to the ligand-blotting analysis in order to localize the bands interacting with 125I-29-kDa fragment. The numerals on the top of the gels indicate fraction numbers. The letters, S and F, indicate the solubilized membrane proteins before fractionation and the proteins that did not bind to the column, respectively.

To purify further the 29-kDa fragment-binding proteins, the fractions containing the 66- and 48-kDa polypeptides were pooled and loaded onto a Sepharose column conjugated with the 29-kDa fragment. Proteins bound to the column were eluted with 4 M urea and electrophoresed in the presence of SDS. As shown in Fig. 3A, only two bands of 66 and 48 kDa were evident upon silver-staining. Furthermore, the peak fraction from the column strongly interacted with 125I-29-kDa fragment as revealed by the ligand-blotting analysis (Fig. 3B, lane a). In addition, this binding could be abolished upon treatment of an excess of nonradioactive 29-kDa fragments (lane b). Thus, it appears clear that the purified 66- and 48-kDa proteins are the specific 29-kDa fragment-binding proteins in the myoblast culture preparations.

Myoblast-specific Binding of the 66-kDa Protein to the 29-kDa Fragment—In order to determine the relationship between the 66- and 48-kDa proteins, antibodies directed only to the 66-kDa protein were prepared as described under "Experimental Procedures." The purified 29-kDa fragment-binding proteins were then subjected to immunoblot analysis using anti-66-kDa antiserum. As shown in Fig. 4A, both the 66- and 48-kDa proteins interacted with the antiserum. These results
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FIG. 4. Cross-reactivity of anti-66-kDa protein antiserum with the 48-kDa protein. Antibody directed to the 66-kDa protein was prepared as described under "Experimental Procedures." A, the purified 29-kDa fragment-binding proteins from the 29-kDa fragment-affinity column (fraction numbers 2-5 in Fig. 3A) were pooled, and aliquots of them (2 μg) were electrophoresed in a 10% slab gel containing SDS. The gel was then subjected to immunoblot analysis using anti-66-kDa antiserum. B, cell lysates were prepared from myogenic cell lines of C2A3 (lane d) and L5E63 (lane e) and fibroblasts from chick embryonic muscles (lane b) and dorsal skin tissues (lane c). The lysates were then electrophoresed and immunoblotted as above. The lysates from primary culture of chick myoblasts were also analyzed as a control (lane a). The blots were visualized by incubation with horseradish peroxidase-conjugated anti-rabbit IgG.

raised possibilities that the 48-kDa polypeptide could be the proteolytic cleavage product of the 66-kDa protein and that either of two proteins might be derived from other contaminating cells, such as fibroblasts, because primary cultures of chick myoblasts could not be completely devoid of fibroblast contamination.

To test these possibilities, cell lysates were prepared from two myogenic cell lines, mouse C2A3 and rat L5E63, and subjected to immunoblot analysis (33). As shown in Fig. 4B, anti-66-kDa antiserum interacted only with the 66-kDa protein in both the cells. On the other hand, the same antiserum reacted solely with the 48-kDa protein when the lysates of chick embryonic fibroblasts were analyzed. Therefore, it appears clear that the 48-kDa protein originates from contaminating fibroblasts in the primary culture of chick myoblasts and the 66-kDa protein is a unique myoblast surface binding protein for the N-terminal 29-kDa fragment of fibronectin. However, the chemical basis for the interaction of anti-66-kDa antiserum with the 48-kDa protein remains unclear.

The 66-kDa Protein Is Distinct from β1-Integrin—To determine whether the 66-kDa protein is distinct from the integrin-type receptor, myoblast lysates were prepared, incubated with anti-β1-integrin antiserum, and precipitated by adding protein A-Sepharose. Both the resulting supernatant and pellet fractions were electrophoresed in the presence of SDS and incubated with the anti-β1-integrin antiserum or anti-66-kDa antiserum and then with anti-rabbit IgG conjugated with horseradish peroxidase. As shown in Fig. 5A, the immunoprecipitation procedure almost completely eliminated from the cell lysates the 120-kDa protein that could interact with anti-β1-integrin antiserum. On the other hand, the 29-kDa fragment-binding proteins that can interact with anti-66-kDa antiserum remained in the supernatant fractions (Fig. 5B). These results indicate that the 66-kDa protein is different from the integrin-type receptor.

Involvement of the 66-kDa Protein in Fibronectin Matrix Assembly—To determine whether the 66-kDa proteins on the surface of chick myoblasts involve matrix assembly of fibronectin, we first examined the effect of anti-66-kDa IgG on the binding of the 29-kDa fragment to the cultured myoblasts. The cells cultured for 30 h were incubated for 2 h with 125I-29-kDa fragment in the presence of anti-66-kDa IgG or preimmune IgG. After the incubation, the cells were solubilized with 4% SDS. Fig. 6 shows that anti-66-kDa IgG inhibits the binding of the 29-kDa fragment in a dose-dependent manner unlike the preimmune IgG. These results further support our finding that the 66-kDa protein is responsible for the interaction with the N-terminal 29-kDa fragment of fibronectin.

We then examined the effect of anti-66-kDa IgG on the binding of fibronectin and its incorporation into fibronectin matrix. The cells cultured for 30 h were incubated for 3 h with 125I-fibronectin and anti-66-kDa IgG. After the incubation, proteins were extracted by treating the cells with 1% deoxycholate and then with 4% SDS. Fibronectin bound to cell surface is known to be extractable by 1% deoxycholate, whereas the protein molecules that were assembled into extracellular matrices can be extracted with 4% SDS but not by 1% deoxycholate (7). As shown in Fig. 7, anti-66-kDa IgG revealed little or no effect on the binding of fibronectin on the cell surface, but markedly inhibited the assembly of the protein into matrices.

To determine whether the exogenously added fibronectin can indeed be incorporated into fibronectin matrices, fluorescence microscopic studies were performed using the cultured myoblasts. As shown in Fig. 8A, incubation with FITC-labeled fibronectin resulted in the formation of an extensive extracellular meshwork that completely surrounded the cells. Furthermore, a similar pattern of meshwork was formed by the cells that were treated with the cells without the exogenously added fibronectin, as analyzed by the sequential treatment of anti-fibronectin IgG and anti-rabbit IgG conjugated with FITC (Fig. 8B). These results clearly show that cultured myoblasts can make fibronectin matrices by themselves as well as they can incorporate exogenous fibronectin into matrices. We then examined whether the fibronectin matrices co-localize with the 66-kDa protein. The cells cultured for 30 h were double-labeled by sequential treatments of FITC-labeled fibronectin, anti-66-kDa Fab fragments, and anti-rabbit IgG conjugated with TRITC. Fig. 8, C and D, show that both the labels are localized to the cell surface and particularly concentrated at the edges of the surface where fibronectin appears to incorporate into matrices, suggesting that the 66-kDa protein co-localizes with the site of fibronectin matrix formation.

In order to clarify further the involvement of the 66-kDa protein in fibronectin matrix assembly, the cells were incubated
fragment in the presence of increasing amounts of anti-66-kDa fragment for 30 h were incubated for 2 h with specific activity of radioiodinated 29-kDa fragment was or preimmune Fab fragments. Thus, it appears likely that the interaction of the N-terminal 29-kDa domain of fibronectin with the 66-kDa protein is necessary for the matrix assembly.

**DISCUSSION**

We have previously demonstrated that the N-terminal 29-kDa fragment of fibronectin binds to the surface of cultured chick myoblasts with an apparent dissociation constant of $1.4 \times 10^{-7}$ M (22). The density of the binding site on the cell surface has also been estimated to be approximately $3.4 \times 10^7$ cell cultured for 30 h. In the present studies, we demonstrate the presence of a myoblast surface protein that is responsible for the binding to the 29-kDa fragment of fibronectin. This protein with an apparent molecular mass of 66 kDa can be not only cross-linked to the 29-kDa fragment but also purified by the affinity column that is covalently conjugated with the fragments.

Noteworthy is, however, the finding that an additional 48-kDa protein also interacts with the 29-kDa fragment and can be co-purified from the myoblast cultures. Furthermore, the 48-kDa protein cross-reacts with antibody raised against the 66-kDa protein. Therefore, we initially suspected whether the 48-kDa protein is derived by proteolytic cleavage of the 66-kDa protein. However, we could demonstrate that the 48-kDa protein is derived from contaminating fibroblasts in the primary culture of chick myoblasts because only the 66-kDa protein is found in the myogenic cell lines of mouse C2 and rat L8E63, while the 48-kDa protein is exclusively found in the cultures of chick embryonic muscle and skin fibroblasts. Nevertheless, it remains totally unclear how the antibody directed against the 66-kDa protein can cross-react with the 48-kDa protein, although it is tempting to speculate that the surface protein may contain a consensus sequence for the specific interaction with the N-terminal 29-kDa fragment of fibronectin.

Several lines of evidence suggest that the 66-kDa protein is a potential candidate for fibronectin matrix assembly receptor. 1) Anti-66-kDa IgG strongly inhibited the interaction of 125I-29-kDa fragment of fibronectin with the antibody directed against the 66-kDa protein in the matrix assembly of fibronectin. 2) Anti-66-kDa IgG inhibited the incorporation of fibronectin to deoxycholate-insoluble fraction in a dose-dependent manner, in contrast to its insignificant effect on the initial binding of fibronectin to the surface of cultured myoblasts. In addition, the immunoprecipitation experiment revealed that the 66-kDa protein does not cross-react with the anti-β1-integrin antisera. Since antibodies against the β1-subunit are known to co-precipitate the α5-subunit, the 66-kDa protein appears to be distinct at least from the α5β1-integrin.

These findings suggest that the binding of fibronectin in the presence of anti-66-kDa IgG is mediated by the interaction of the cell-adhesive domain of the protein molecule with integrin but not by the interaction of the 29-kDa fragment with the 66-kDa protein. 2) Anti-66-kDa IgG inhibited the incorporation of fibronectin to deoxycholate-insoluble fraction in a dose-dependent manner, in contrast to its insignificant effect on the initial cell surface binding. McKeown-Longo and Mosher (7) have previously shown that, using pulse-chase experiments, fibronectin binds initially in the deoxycholate-soluble fraction (cell surface fraction) and is transferred to the deoxycholate-insoluble fraction (extracellular matrix). Therefore, it appears that anti-66-kDa IgG prevents the assembly of fibronectin into matrices by blocking the interaction of the N-terminal 29-kDa fragment of fibronectin with the 66-kDa receptor. 3) Perhaps the most compelling evidence for involvement of the 66-kDa protein in the matrix assembly of fibronectin are the fluorescence microscopic studies demonstrating that the incubation time-dependent incorporation of FITC-labeled fibronectin can be completely prevented by treatment of the anti-66-kDa Fab fragments to the cultured myoblasts. In addition, the 66-kDa protein appears to co-localize with the site of fibronectin matrix formation. Therefore, fibronectin matrix assembly appears to be mediated by the interaction of the N-terminal type I domain.

**Fig. 6.** Effect of anti-66-kDa IgG on the binding of 125I-29-kDa fragment of fibronectin to cultured myoblasts. The cells cultured for 30 h were incubated for 2 h with 1.4 x 10^5 cpm/ml of 125I-29-kDa fragment in the presence of increasing amounts of anti-66-kDa IgG () or preimmune IgG (○). After the incubation, they were solubilized with 4% SDS and counted for radioactivity. The amounts of 125I-29-kDa fragment bound were then expressed as picograms/5 x 10^5 nuclei. The specific activity of radiiodinated 29-kDa fragment was 2 x 10^6 cpm/µg. The values represent the mean ± standard error of five independent experiments.

**Fig. 7.** Effect of anti-66-kDa IgG on the surface binding of 125I-fibronectin and its incorporation into deoxycholate-insoluble matrices. Chick myoblasts cultured for 30 h were incubated for 3 h with 1.3 x 10^5 cpm/ml of 125I-fibronectin in the presence of increasing amounts of anti-66-kDa IgG. After the incubation, proteins from the cells were extracted with 1% deoxycholate (○) and then with 4% SDS (●). The resulting extracts were counted for radioactivity. The amounts of 125I-fibronectin bound were then expressed as nanograms/5 x 10^5 nuclei. The specific activity of radiiodinated fibronectin was 1.1 x 10^6 cpm/µg. The values represent the mean ± standard error of five independent experiments.

for different periods with FITC-labeled fibronectin in the presence of Fab fragments of anti-66-kDa protein IgG or preimmune IgG. As shown in Fig. 9, A and B, the cells incubated for 15 min with anti-66-kDa Fab fragments were capable of binding with FITC-labeled fibronectin as well as those incubated with preimmune Fab fragments. Thus, it appears likely that the 66-kDa protein is not involved in the initial binding of fibronectin to the cell surface. When the cells were incubated for 3 h, however, anti-66-kDa Fab fragments completely blocked the incorporation of FITC-labeled fibronectin into matrices (Fig. 9D), unlike the preimmune Fab fragments (Fig. 9C). These results strongly suggest that the interaction of the N-terminal 29-kDa domain of fibronectin with the 66-kDa protein is necessary for the matrix assembly.
Fig. 8. Incorporation of exogenously added fibronectin into matrices and its co-localization with the 66-kDa protein. Chick myoblasts cultured for 30 h were incubated for 3 h in the presence and absence of 200 μg/ml of FITC-labeled fibronectin, rinsed with DMEM, and further incubated in DMEM containing 10% horse serum for the next 24 h. After the incubations, the cells incubated with FITC-labeled fibronectin were fixed and observed as described under “Experimental Procedures” (A). The cells incubated in DMEM containing 10% horse serum but without FITC-labeled fibronectin were fixed, treated sequentially with anti-fibronectin IgG and anti-rabbit IgG conjugated with FITC, and observed (B). The cells cultured for 30 h were also incubated for 30 min with FITC-labeled fibronectin (C). The resulting cells were fixed, treated with the anti-66-kDa IgG and then with anti-rabbit IgG conjugated with TRITC, and observed (D). Bar, 20 μm.

Fig. 9. Effect of Fab fragments of anti-66-kDa IgG on the surface binding of FITC-labeled fibronectin and its incorporation into fibronectin matrices. To determine the cell surface binding of fibronectin, chick myoblasts cultured for 30 h were incubated for 15 min with 200 μg/ml of FITC-labeled fibronectin in the presence of 120 μg/ml of Fab fragments of preimmune IgG (A) or anti-66-kDa IgG (B) in DMEM supplemented with 10% fibronectin-depleted horse serum. When assayed for the incorporation into fibronectin matrices, the cells were incubated for 3 h with FITC-labeled fibronectin in the presence of Fab fragments of preimmune IgG (C) or anti-66-kDa IgG (D). The cells were washed with DMEM containing 10% horse serum and further cultured for the next 24 h. The cultures were then fixed and observed as in Fig. 8. Bar, 20 μm.

of fibronectin with its 66-kDa receptor on the surface of cultured myoblasts.

A number of reports have suggested that other regions in fibronectin also involve in matrix assembly. Monoclonal antibodies directed to the RGD-containing cell-adhesive domain of fibronectin were found to inhibit matrix assembly (34). The antibodies against the integrin that binds to the RGD site in fibronectin were also found to inhibit fibronectin matrix assembly (35, 36). In addition, a 14-kDa fragment containing the first two type III repeats of fibronectin was shown to inhibit fibronectin matrix assembly (37). The presence of multiple cell-binding regions in fibronectin has been explained by the multistep model for fibronectin matrix assembly, proposed by Schwarzbauer et al. (38). Similar models have also been proposed by McDonald (10) and Mosher et al. (39). This model involves four sequential events: capture of fibronectin mol-
ecules from the environment, translocation of the captured fibronectin to the growing end of fibronectin fiber, alignment of fibronectins, and, finally, covalent stabilization of the fibronectin fibril. Therefore, it is possible that the 66-kDa protein is involved in the later event in the first step for fibronectin capture, since the anti-66-kDa IgG inhibits significantly the initial binding of fibronectin but remarkably the incorporation of fibronectin into deoxycholate-insoluble matrices.

The role(s) of fibronectin and its assembly into extracellular matrices on the regulation of differentiation of cultured myoblasts remains unknown. However, a number of reports have suggested the involvement of fibronectin in the myogenic process (for review, see Ref. 19). Myoblasts have been reported to adhere to fibronectin (40) and extend along the oriented fibrils of fibronectin (41). It was also reported that fibronectin-coated substratum promotes fusion although it does not distinguish between effects on adhesion and on fusion per se (42). Also reported was that the amount of cell surface fibronectin decreases after the fusion of myoblasts into myotubes (43). In addition, we have recently demonstrated that the decrease in the level of fibronectin during myoblast fusion is closely correlated with the gradual loss of the binding activity of the cell surface with the 29-kDa fragment (22) and that the expression of the 66-kDa protein falls dramatically during the fusion process. However, more studies are necessary for understanding the precise action mode of fibronectin and the mechanism by which the expression of the 66-kDa receptor is regulated during the differentiation of chick embryonic myoblasts.

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