Relationship of Heparan Sulfate Proteoglycans to the Cytoskeleton and Extracellular Matrix of Cultured Fibroblasts

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ABSTRACT The distribution of heparan sulfate proteoglycans (HSPG) on cultured fibroblasts was monitored using an antiserum raised against cell surface HSPG from rat liver. After seeding, HSPG was detected by immunofluorescence first on cell surfaces and later in fibrillar deposits of an extracellular matrix. Cell surface HSPG aligned with microfilament bundles of rat embryo fibroblasts seen by phase-contrast microscopy but was diffuse on transformed rat dermal fibroblasts (16C cells) which lack obvious stress fibers. Focal adhesions isolated from either cell type and monitored by interference reflection microscopy showed a concentration of HSPG labeling with respect to the rest of the membrane. Increased labeling in these areas was also seen for fibronectin (FN) by using an antiserum that detects both plasma and cell-derived FN.

Double immunofluorescent staining of fully adherent rat embryo fibroblast cells showed some co-distribution of HSPG and FN, and this was confirmed by immunoelectron microscopy, which detected HSPG at localized areas of dorsal and ventral cell membranes, overlapping cell margins, and in the extracellular matrix.

During cell shape changes on rounding and spreading, HSPG and FN may not co-distribute. Double labeling for actin and either HSPG or FN showed a closer correlation of actin with HSPG than with FN. The studies are consistent with HSPG being closely involved in a transmembrane cytoskeletal-matrix interaction; the possibility that HSPG coordinates the deposition of FN and other matrix components with cytoskeletal organization is discussed.

Fibroblasts in vitro synthesize and secrete a range of glycoproteins and proteoglycans which are present on cell surfaces and deposited around cells to form an extracellular matrix (9, 24, 26, 27, 54, 59). Since the amounts and types of proteoglycans produced by cells have been observed to vary with cell behavior, these molecules have been implicated in the control of cell growth (32, 46), locomotory (18, 21), and adhesive activities (7, 15, 35) governing differentiation and tissue organization (12). Normal cells deposit a dense matrix whereas transformed cells produce much less extracellular matrix (20, 24, 27, 54, 57), with a reduction in heparan sulfate proteoglycan (HSPG)[1] deposition (24). This in conjunction with observed changes in HSPG synthesis on transformation (11, 17, 19, 55, 58) implicates HSPG as a control factor also in the assembly of extracellular matrix through its ability to interact with other matrix components such as fibronectin (FN) and collagens (30, 31, 36, 39, 43, 47, 51).

FN has been shown to be a potent mediator of fibroblast adhesion to substrata (27, 42, 59) and to be a component of

[1] *Abbreviations used in this paper:* FN, fibronectin; HSPG, heparan sulfate proteoglycans; REF, rat embryo fibroblasts.

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transmembrane assemblies with microfilaments (2, 28, 50) which may regulate the organization of both the intracellular cytoskeleton and extracellular matrix. This interaction appears crucial for growth of anchorage-dependent fibroblasts (4, 52). Recently, cell surface HSPG has also been implicated in cell–substratum adhesion of fibroblasts (37).

We report here immunofluorescent and immunoelectron microscopic studies on the cell surface and extracellular matrix distributions of HSPG and FN in normal and transformed rat fibroblasts. These studies demonstrate a co-distribution of HSPG and FN, co-linearity with the internal actin stress fibers of normal cells, and the presence of both matrix components at isolated focal adhesions (1, 3, 25, 29) of fully adherent normal and transformed fibroblasts. The co-distribution of FN and HSPG does not, however, persist under conditions of different microfilament organization such as during cell spreading or detachment; the two matrix components in these circumstances differ in distribution and hence possibly in function.

MATERIALS AND METHODS

Cells: Culture and Experimental Conditions

Culture conditions for rat embryo fibroblasts (REF; Flow Laboratories, Irvine, Scotland) and 16C cells (Colworth strain of an established cell line of spontaneously transformed rat dermal fibroblasts) were as previously described (3, 6). For light and electron microscopy, cells were seeded onto 10-mm-diam glass coverslips or 1 × 2 cm Melinex strips (ICI, Runcorn, Cheshire, U.K.), respectively. Seeding density and time of culture varied as below: (*a*) Fully adherent sparse, confluent, or dense cultures were obtained by seeding at 3.5×10^4 cells/cm^2 (REF) or 5.0×10^4 cells/cm^2 (16C) and incubating these 1, 2, or 3 d, respectively. (*b*) Focal adhesions were prepared 2 d after seeding at 7.0×10^4 cells/cm^2 (REF) or 10.0×10^4 cells/cm^2 (16C). (*c*) Studies of cell rounding were performed on sparse REF cultures. (*d*) Studies of cell spreading used 2.0×10^5 REF cells/cm^2 incubated 1–1.5 h. (*e*) Radiolabeling was performed by adding 10 ml of medium containing [^{35}S]sulfate (100 μCi/ml) or [^{3}H]glycine (10 μCi/ml) (Amersham International, Bucks, U.K.) to 3-d cultures of REF seeded at 1.0×10^6 cells/75-cm^2 culture flask (Corning Glass Works, Stone, Staffordshire, U.K.) and incubating a further 3 d by which time the cultures were highly dense. Focal adhesions of 16C cells were prepared using a stream of PBS (Flow Laboratories) as previously described (3). REF cultures were preincubated at 4°C for 15 min to facilitate preparation of subunit focal adhesions similar to those previously reported for these cells after EGTA treatment (6). In studies of cell rounding, live cells were incubated with 10 μg/ml trypsin (Sigma type III; Sigma Chemical Co., Poole, Dorset, U.K.) in PBS or 0.05% EGTA (Sigma Chemical Co.) in Ca^{2+}-, Mg^{2+}-free PBS (Flow Laboratories) for 2–10 min at 37°C.

Fluorescence Staining

For indirect immunofluorescence cells were fixed with 3.5% paraformaldehyde as previously described (3, 4, 6) followed, when permeabilization was needed, by extraction with 0.1% Triton X-100 in PBS for 10 min at room temperature and three washes (10 min each) in PBS. Cells were incubated with antiserum for 45 min at 37°C followed by three washes (15 min each) with PBS. It was found that labeling of HSPG and FN under spread cells was increased after permeabilization, and this was routinely performed unless otherwise stated. Double immunofluorescence was performed by simultaneous addition of both primary antisera followed by simultaneous addition of the appropriate fluorochrome-conjugated antisera. Controls were routinely performed to detect any nonspecific staining or cross-reactivities between inappropriate antisera: addition of both second antisera with either first antiserum showed only the expected fluorescence, addition of the inappropriate second antiserum gave no labeling, and second antisera alone gave no fluorescence. Actin was labeled with phalloidin coupled to rhodamine (a gift from Dr. R. Warn, University of East Anglia, Norwich, U.K.) diluted to 0.4 μg/ml in PBS.

Antisera

Rabbit anti-HSPG serum was prepared by immunization with rat liver HSPG, prepared as previously described (41). Proteoglycans corresponding to 25 μg protein (in 100 μl) was emulsified with 100 μl of Freund's complete adjuvant and injected as 50-μl aliquots into a lymph node on the back of each thigh. Booster doses with incomplete adjuvant were given twice at 2-wk intervals. The rabbits were bled 10, 20, and 30 d after the last injection. The immunoreactivity of the antiserum was tested by precipitation of [^{35}S]sulfate-labeled proteoglycans with Protein A-Sepharose. The rabbit antiserum has been characterized and found to be specific for the core protein of HSPG (Kjellén, L., and M. Höök, manuscript in preparation). For immunofluorescent staining this antiserum and another antiserum to HSPG from murine EHS tumor (23, 24) (a gift from Dr. J. R. Hassell, National Institutes of Health) were both used at 1:5 dilution in PBS. Antigen absorption of the anti-HSPG serum was as follows. The intercalated form of HSPG was extracted with Triton X-100 from plasma membrane fractions of hepatocytes after removal of the peripheral HSPG by incubation with heparin (34). This was purified by ion exchange chromatography and gel filtration on a column of Sepharose CL-4B as previously described (34). Purity of the antigen was confirmed by iodination of the preparation followed by autoradiography which detected no ^{125}I-labeled components other than HSPG (Kjellén, L., and M. Höök, manuscript in preparation). 60 μl (6 μg) of HSPG in 0.05 M sodium acetate, pH 4.0, 1.5 M NaCl, 0.1% Triton X-100 was incubated with 12 μl of anti-HSPG serum for 1 h at 37°C. The mixture was centrifuged at 3,000 *g* for 10 min and the supernatant was used as primary antiserum in immunofluorescent staining.

Guinea pig antiserum to bovine FN was obtained 1 mo after immunization with 1 × 2 mg of bovine plasma FN (a gift from Dr. S. Chavin, Rochester General Hospital, N. Y. [14]) in Freund's complete adjuvant. This antiserum gave a single precipitin line in Ouchterlony diffusion plates against bovine plasma FN, and its immunofluorescent staining activity on rat cells could be removed by preadsorption with bovine and rat FN coupled to Sepharose. Since it is reactive to bovine plasma FN, this antiserum would stain plasma FN absorbed to cells under our culture conditions. It also stains REF cell-derived FN when cells are seeded in serum-free conditions (not shown). Thus it detects total FN from both sources in our systems. It was used at 1:50 dilution in PBS.

Second antisera were F(ab')$_2$ fragments of goat anti-guinea pig IgG conjugated with fluorescein isothiocyanate (Cappel Laboratories, Cochranville, PA) and goat anti-rabbit IgG conjugated with fluorescein isothiocyanate or tetramethylrhodamine isothiocyanate (Miles Laboratories Inc., Slough, Bucks, U.K.). All were used at 1:50 dilution in PBS.

Light Microscopy

Cells were viewed on a Leitz Ortholux II microscope fitted with epifluorescence, phase-contrast, and interference reflection objectives. No breakthrough of the rhodamine image into the fluorescein image or vice versa was detected at the levels of antisera and phalloidin used. Photographs were taken on Ilford HP5 films with exposures of 30–120 s, the fluorescein image being exposed first.

Immunoelectron Microscopy

Cells were treated sequentially with 1% paraformaldehyde/0.05% glutaraldehyde (Emscope, Ashford, Kent, U.K.) in PBS (20 min), PBS (15 min), 0.1 M NH_4Cl in PBS (15 min), and 1% ovalbumin (Sigma) in PBS (15 min). Incubation with anti-HSPG (45 min at 37°C) was followed by three washes (15 min each) with PBS and incubation (45 min at 37°C) with 5-nm colloidal gold–goat anti-rabbit complex (Janssen Pharmaceutica, Beerse, Belgium) at 1:20 dilution in 0.45 M NaCl, 0.02 M Tris-HCl, pH 7.2, 0.5 mg/ml polyethylene glycol (20K; Sigma Chemical Co.). Samples were washed in PBS (three washes, 15 min each), fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2, mixed with cacodylate buffer, and postfixed (30 min) with cacodylate buffer containing 1% osmium tetroxide and 0.5 mg/ml ruthenium red (TAAB, Reading, U.K.) to stain glycocalyx components. Cells were then washed with running distilled water (15 min), dehydrated through a graded ethanol series, and embedded as previously described (6). To enhance contrast and visualization of matrix staining by ruthenium red, uranyl acetate and lead citrate poststaining were omitted.

Analysis of Radiolabeled Medium from Cells

15 ml of medium from [^{35}S]sulfate-labeled cells was added to a 1-ml column of DEAE-Sephacel equilibrated in 0.2 M NaCl, 0.05 M Tris buffer, pH 8.0. The column was subsequently washed with (*a*) 10 ml of 0.2 M NaCl, 0.05 M Tris buffer, pH 8.0, and (*b*) 10 ml of 0.2 M NaCl, 0.05 M acetate buffer, pH 4.0, and eluted with 2 ml of 1.5 M NaCl, 0.05 M acetate buffer, pH 4.0. The material eluted from the column is enriched in ^{35}S proteoglycans whereas unincorporated $^{35}SO_4$ and most proteins are lost in the washings. Before further

experiments, the purified ^{35}S macromolecules were dialyzed against PBS containing 0.5 M NaCl.

The ^{3}H-labeled macromolecules were separated from unincorporated [^{3}H]-glycine by gel chromatography on a 10-ml Sephadex G-25 (PD-10) column, eluted with PBS containing 0.5 M NaCl. Fractions containing the ^{3}H-labeled proteins were pooled and concentrated by ultrafiltration.

IMMUNOPRECIPITATION: ^{35}S-labeled macromolecules or ^{3}H-labeled macromolecules (150,000 cpm) were incubated overnight with 50 μl of antiserum (anti-HSPG serum, anti-rat FN serum, or control serum) in a total volume of 0.5 ml in PBS containing 0.5 M NaCl. 200 μl of Protein A-Sepharose suspension (corresponding to 100 μl of packed gel) was added and the incubation continued for 1 h at room temperature. After centrifugation at 3,000 rpm for 5 min, the gel was washed three times with 2 ml of PBS containing 0.5 M NaCl. The gel was eluted by boiling the samples for 3 min in 200 μl of 4 M guanidine-HCl, followed by centrifugation for 5 min at 3,000 rpm. The supernatant was collected and 100 μl of it was assayed for radioactivity as was the remaining gel which still contained some radioactivity. The remaining 100 μl of the supernatant was dialyzed against water, digested with papain as previously described (41), and subjected to nitrous acid deamination at pH 1.5 (49) to estimate the content of heparan sulfate. The samples were analyzed on a Sephadex G-50 column and eluted with 1 M NaCl, and the percentage of the total radioactivity eluting in the retarded peak near the total volume of the column was classified as heparan sulfate.

RESULTS

To analyze the specificity of the anti-HSPG serum, we used it to immunoprecipitate ^{35}S- and ^{3}H-labeled macromolecules isolated from the medium of REF cells incubated with [^{35}S]sulfate and [^{3}H]glycine, respectively. Polyanionic ^{35}S-labeled macromolecules were purified by anion exchange chromatography and consisted of 27% HSPG as determined by susceptibility to nitrous acid deamination.

After incubation of the ^{35}S-labeled macromolecules with anti-HSPG serum followed by Protein A-Sepharose, 8.5% of the total ^{35}S radioactivity was associated with the gel, and thus bound to IgG (Table I). The gel-bound radioactivity was present in heparan sulfate as shown in Fig. 1. Hence, of the different proteoglycans produced by fibroblasts, only a portion of the HSPG was precipitated with the antiserum.

We explored the possibility that the antiserum reacts with other proteins by testing the antiserum against [^{3}H]glycine-labeled proteins obtained from medium over REF cells. As can be seen from Table I, 5% of the ^{3}H-labeled proteins react with antiserum against rat FN, while 1.8% of the radioactivity reacted with control serum, and slightly more with anti-HSPG serum. Anti-HSPG does not react with [^{35}S]methionine-labeled FN synthesized by REF cells in culture (data not shown). These results suggest that the anti-HSPG serum does not react with REF products other than the HSPG.

The immunofluorescent staining on confluent cultures of

TABLE I

Immunoprecipitation with Protein A-Sepharose of [^{35}S]Sulfate- or [^{3}H]Glycine-labeled Medium Macromolecules from REF Cells

Label	Present in heparan sulfate	Radioactivity: Binding to anti-HSPG IgG	Binding to control IgG	Binding to anti-FN IgG
			%	
[^{35}S]Sulfate*	27	8.5	0.6	ND
[^{3}H]Glycine‡	ND	2.2	1.8	5.2

* Purified by DEAE-ion exchange chromatography as described in Materials and Methods.

‡ Purified by gel chromatography on Sephadex G-25 as described in Materials and Methods.

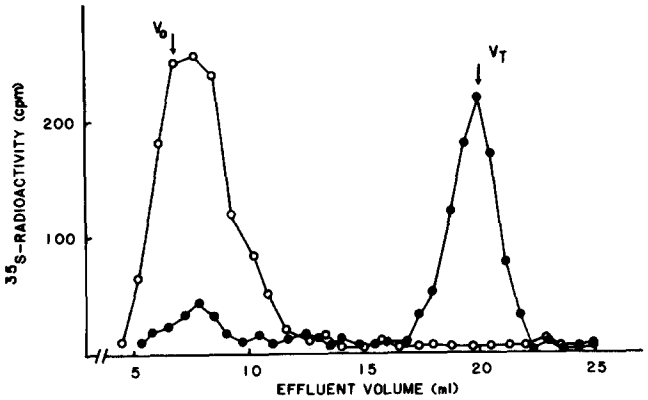

FIGURE 1 Heparan sulfate nature of the anti-HSPG immunoprecipitate. The [^{35}S]sulfate-labeled anti-HSPG immunoprecipitate eluted from the Protein A-Sepharose with 4 M guanidine-HCl and was dialyzed against water and subjected to papain digestion. The digested sample was analyzed on a Sephadex G 50 column (20 ml) eluted with 1 M NaCl before (○) and after (●) nitrous acid deamination. Fractions of 0.7 ml were collected and assayed for ^{35}S radioactivity.

REF cells seen with antiserum raised against cell surface rat liver membrane HSPG (anti-HSPG) is shown in Fig. 2*a*, together with that seen (Fig. 2*b*) when a previously reported antiserum against basement membrane HSPG (anti-BM_1) was used (24, 26). Anti-HSPG stains extracellular and cell surface matrix fibrils (arrows) together with some more diffuse cell surface staining (see below) and this is greatly reduced by prior absorption of the antiserum with HSPG extracted with Triton X-100 from rat liver (34) (Fig. 2*c*) but not by prior absorption with rat or bovine FN (not shown). In contrast, FN labeling with guinea pig antibovine FN was reduced by prior absorption with rat or bovine FN but not with HSPG (not shown).

HSPG Distribution

The distribution of HSPG at different cell densities of normal and transformed cells is illustrated in Fig. 3. Sparse REF cells which have very small amounts of fibrillar extracellular matrix (Fig. 3*a*, arrowhead) showed diffuse HSPG membrane staining with concentrations of labeling over stress fibers visible by phase-contrast microscopy (Fig. 3*b*). Membrane concentrations of label for HSPG sometimes appeared "condensed" into discrete fibrils co-linear with stress fibers (Fig. 3, *a* and *b*, arrows). These cell surface fibrils resemble in size and intensity of staining those previously reported to contain HSPG and FN (9, 24, 26, 28) and may be classed as extracellular matrix rather than increased membrane label concentrations. 16C cells, which do not have stress fibers detectable by phase-contrast microscopy, showed only a diffuse membrane staining (Fig. 3, *d* and *e*). Similar distributions were seen for FN on these cell surfaces (not shown but see references 26, 28, and 50), although for both cell types the amount of FN labeling was greater than HSPG staining. A co-linearity with stress fibers was not observed when fixed cells were labeled for general membrane components (for example concanavalin A receptors [5]). Hence, the co-alignment of HSPG and FN with stress fibers does not represent artifactual labeling due to our fixation and processing protocols. As cells approached confluency and especially in dense

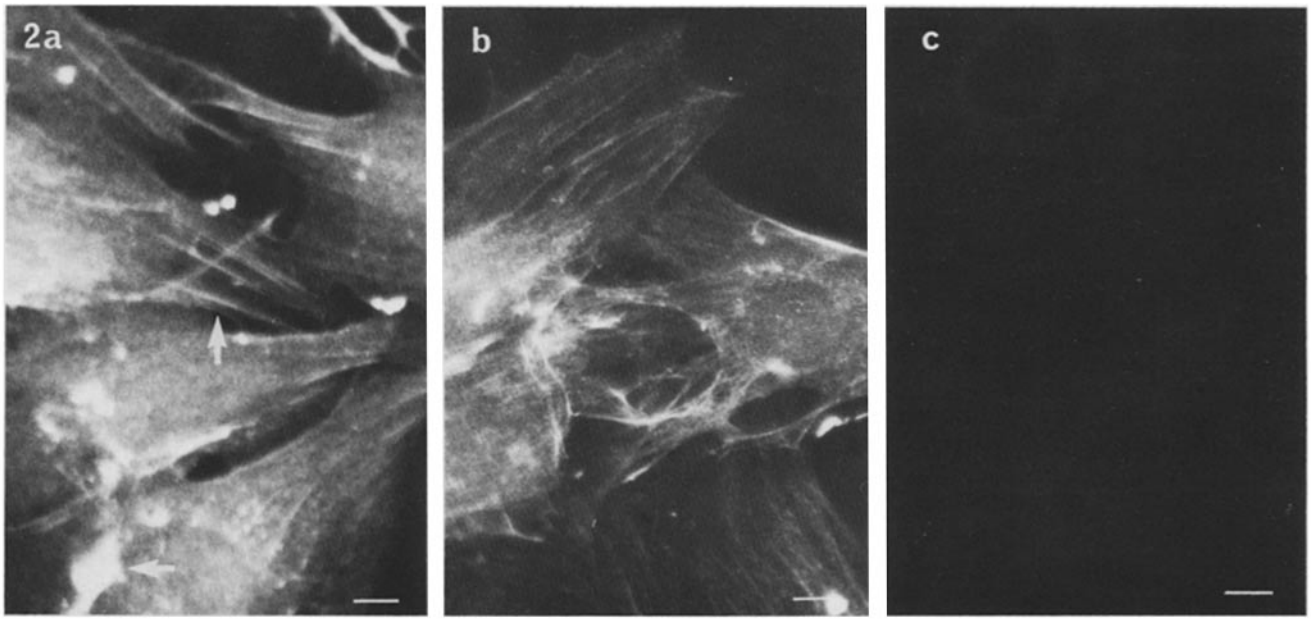

FIGURE 2 Immunofluorescence micrographs of REF cultures labeled with (*a*) anti-HSPG, (*b*) anti-BM_1, and (*c*) anti-HSPG preabsorbed with HSPG. Note that extracellular matrix deposits and fibrils (arrows) and diffuse membrane labeling seen in *a* are absent in *c*. Bars, 10 μm. × 630.

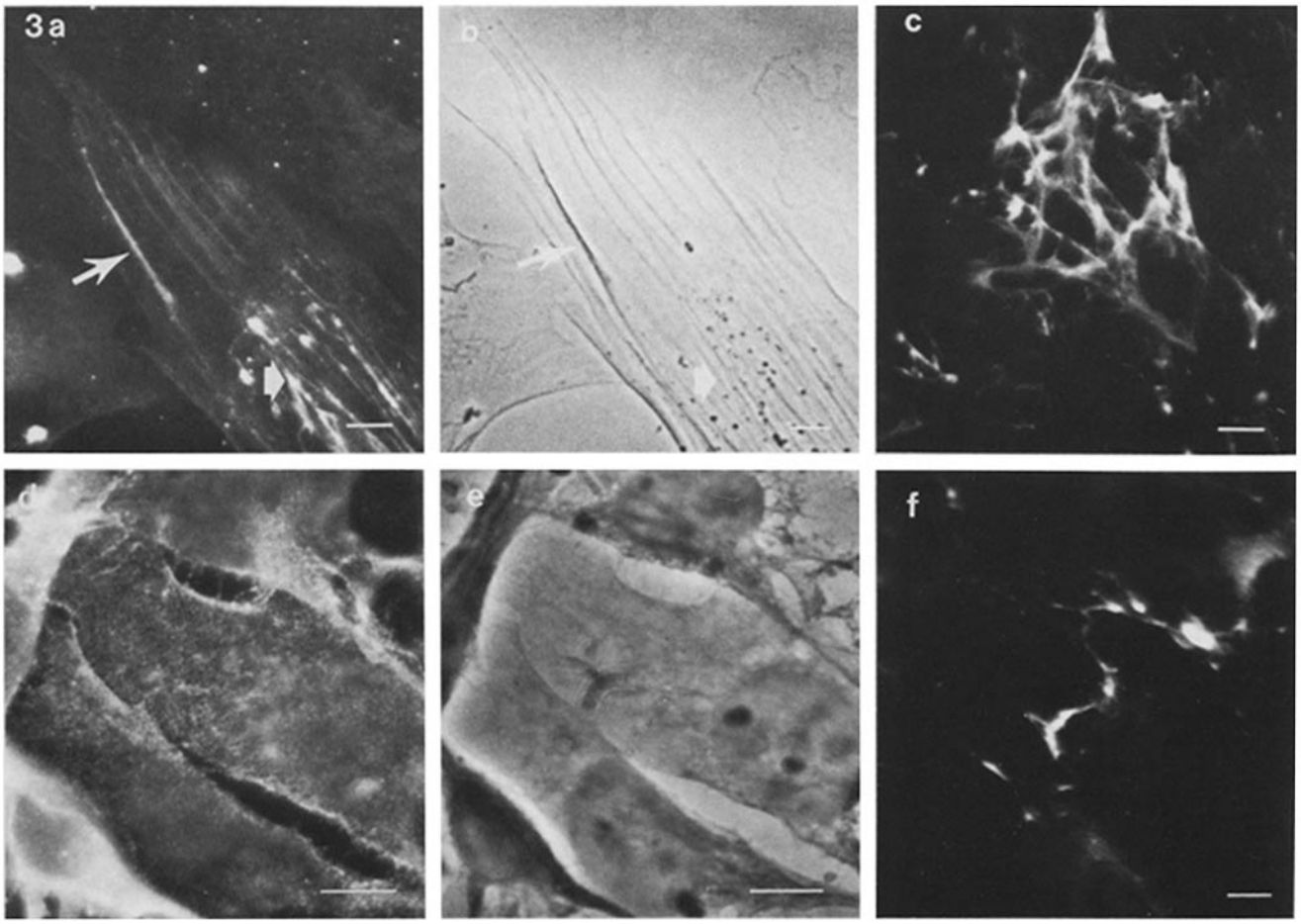

FIGURE 3 Immunofluorescence (*a*, *c*, *d*, and *f*) and phase-contrast (*b* and *e*) micrographs on REF (*a–c*) and 16C cells (*d–f*) labeled with anti-HSPG. HSPG distribution on sparse REF cells (*a*) shows membrane concentrations and cell surface fibrils (arrows) correlating with stress fibers (*b*), in addition to fibrillar deposits over the central cell area (arrowheads). Dense REF cultures (*c*) show HSPG in the extracellular matrix but reduced on the cell surface. 16C cells show diffuse HSPG labeling (*d*) in sparse culture and a small amount of matrix deposition in dense cultures (*f*). Bars, 10 μm. (*a–c*, and *f*) × 630; (*d* and *e*) × 990.

cultures, extracellular matrix fibrils were produced and these fibrils stained for both HSPG (Fig. 3, *c* and *f*) and FN (not shown). The amount of extracellular matrix was much reduced in 16C cultures compared with REF, which is consistent with previous reports that demonstrate reduced matrix deposition by transformed cells (20, 24, 27, 42, 54, 57).

Further correlation of HSPG and FN concentrations with stress fiber distribution was seen by labeling for both components in isolated REF and 16C cell focal adhesions. As previously demonstrated, these structures are present at the termini of actin microfilament bundles (3, 22, 25) and vary in size with cell type (6), normal REF adhesions being much larger than those of 16C cells. Fig. 4 shows isolated adhesions detected by their interference reflection image (*b*, *d*, and *f*) stained for HSPG (*a* and *c*) and FN (*e*). The correlation was very striking, especially in REF cells whose adhesions in intact cells break upon isolation to give subunit adhesions similar to those previously seen after EGTA treatment of this cell type (6); each subunit stained heavily for HSPG and FN. Labeling at adhesions was increased with respect to that of any surrounding membrane pieces, which remained attached to adhesions on isolation and also appeared dark by interference reflection microscopy owing to collapse on to the substrate (Fig. 4, *a* and *b*, arrows). Since the membrane usually found on intact cells at this density also showed little HSPG staining (see Fig. 3, *c* and *f*), these results indicate a concentration of HSPG and FN around adhesion areas. Fibrils of extracellular matrix retained on the substrate after preparation of adhesions also labeled for HSPG and FN (not shown).

Double immunofluorescent labeling of REF cultures with antisera to HSPG and FN showed some co-distribution of these components on both cell surfaces and extracellular matrix fibrils in that the larger fibrils and deposits of FN were also positively labeled for HSPG (Fig. 5, *a–d*) although there were areas of FN staining that labeled to a much lesser extent with anti-HSPG (arrowheads in Fig. 5). This is consistent with recent results using other cell types and an antiserum to basement membrane HSPG (24, 26). During preliminary experiments to perfect the labeling protocol, we noted that addition of anti-FN serum before anti-HSPG serum markedly reduced the level of HSPG labeling when compared with single immunofluorescent labeling or addition of antiserum in the reverse order. This indicated that the interaction of HSPG with FN may be very close and antibody access to HSPG could be limited by a "masking" effect of FN which is further increased by addition of anti-FN.

Immunoelectron microscopy showed HSPG to be present both on REF cell surfaces and in the extracellular matrix deposited by these cells. HSPG was detected at discrete areas on dorsal (Fig. 6*a*) cell membranes, including areas where matrix components are in close contact with the membrane and on ventral cell membranes where it was often in association with fibrillar deposits connecting the cell to the substratum (Fig. 6*b*, arrows). HSPG was also detected between overlapping cell edges (Fig. 6*c*, arrows) and in open fibrillar networks of the extracellular matrix, in particular at the junctions between anastomosing fibrils (Fig. 6*d*, arrows). Dense deposits of extracellular matrix often did not show heavy labeling for HSPG; colloidal gold was limited to the exterior of these structures. This may be a reflection of limited HSPG antibody access similar to the "masking" of collagen to antibodies by other matrix components in chick heart fibroblast cultures (40).

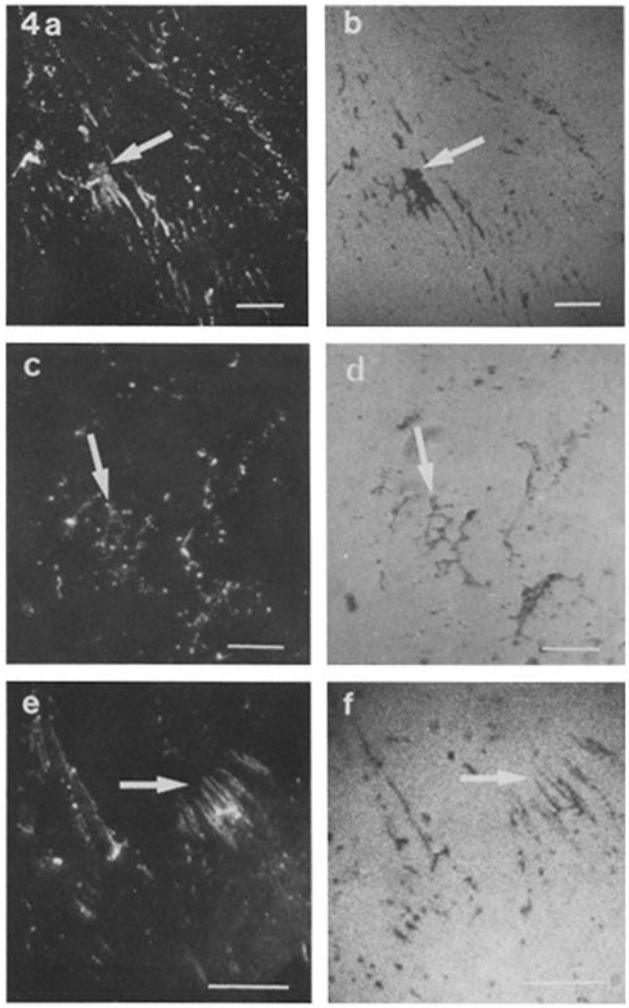

FIGURE 4 Corresponding immunofluorescence and interference reflection micrographs of isolated focal adhesions from REF (*a*, *b*, *e*, and *f*) and 16C (*c* and *d*) cells labeled with anti-HSPG (*a* and *c*) and anti-FN (*e*). Corresponding areas are arrowed in pairs, top, middle, and bottom. Bars, 10 μm. (*a* and *b*) × 420; (*c* and *d*) × 660; (*e* and *f*) × 1,040.

Since both HSPG and FN showed co-linearity and possible coordination of deposition on the cell surface with the organization of the actin cytoskeleton in fully adherent cells, we monitored their distribution under more dynamic conditions of cell rounding and spreading to attempt to dissect the mechanism of organization. Fig. 7 shows double immunofluorescent labeling for HSPG and FN and either matrix component contrasted with the distribution of actin during cell rounding induced by trypsin treatment. Although untreated cultures labeled more heavily for FN than for HSPG, mild trypsin treatment of cells caused a reversal of this situation. HSPG deposits were now detected that had much less corresponding FN label (Fig. 7, *a* and *b*, arrowheads) and HSPG was seen in areas lacking FN staining. This was true for both permeabilized (Fig. 7, *a* and *b*) and nonextracted (not shown) trypsin-treated cells. Other cells under similar treatment conditions were double labeled using phalloidin and antisera to either HSPG or FN. Fig. 7, *c* and *d* show HSPG (Fig. 7*c*) still coincident with some actin stress fibers (Fig. 7*d*, arrows) whereas FN membrane labeling was much reduced even before stress fiber break-up (Fig. 7, *e* and *f*) leaving only

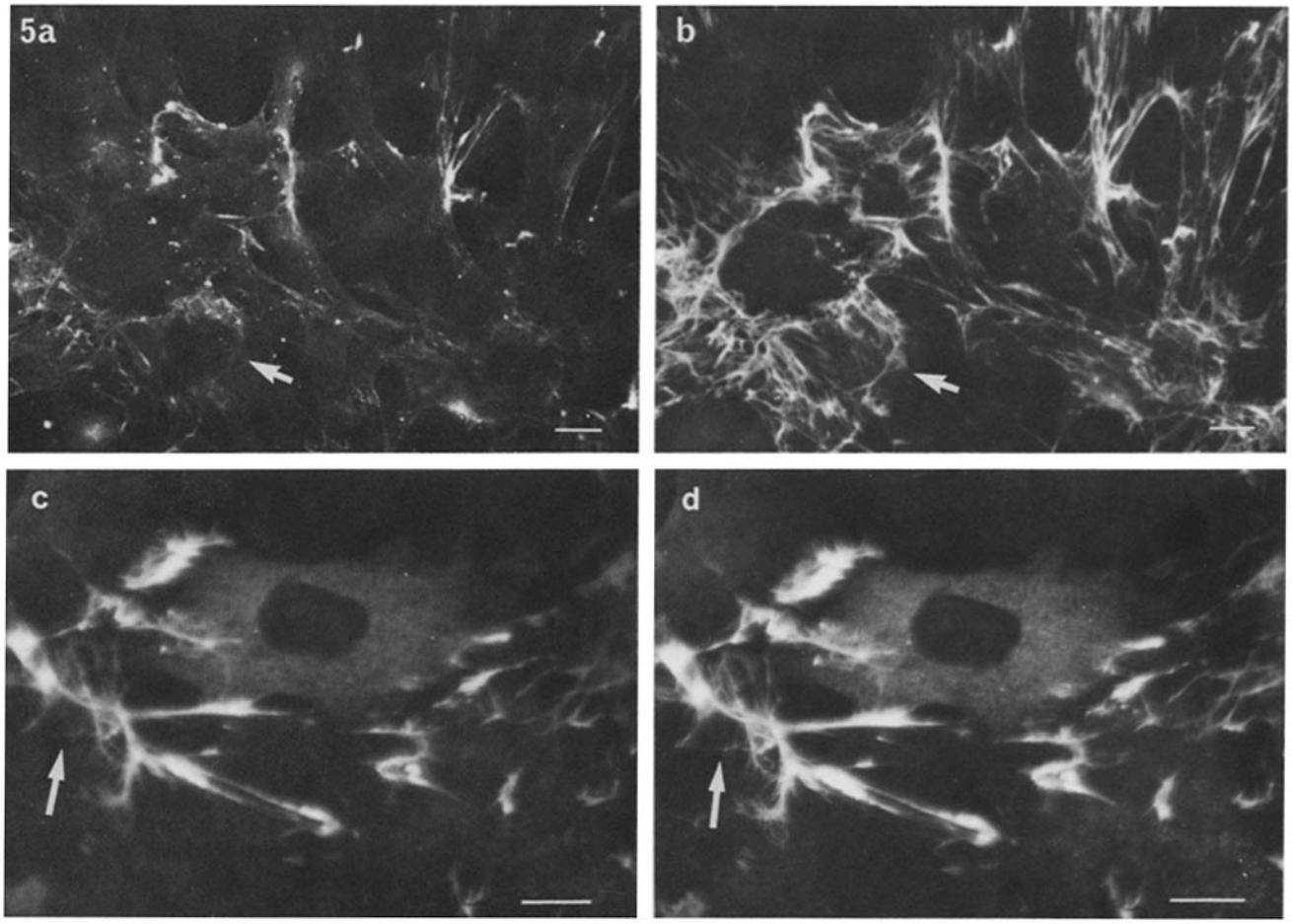

FIGURE 5 Double immunofluorescence micrographs of REF cells labeled with anti-HSPG (a and c) and anti-FN (b and d). Dense (a and b) and confluent (c and d) cultures are shown and arrows indicate areas staining more heavily for FN than for HSPG. Bars, 10 μm. × 630.

intracellular FN staining. The HSPG staining seen on rounding cells often appeared as linear arrays of particulate labeling (Fig. 7*c*, arrow) coincident with stress fibers rather than as complete lines (compare with Fig. 3, *a* and *b*). This was mainly evident in areas where stress fibers were beginning to break up. Rounded cells after prolonged trypsin treatment retained some cell surface HSPG labeling, as shown by the staining of nonextracted cells (Fig. 7*g*), whereas very little external FN label could be detected after trypsin-induced cell rounding (Fig. 7*h*).

Similar results were obtained when cells were induced to round under the action of EGTA (Fig. 8). Again, diffuse cell surface HSPG (Fig. 8*a*) was retained longer on the cell surface than was FN (Fig. 8*b*). Membrane concentrations of HSPG still aligned with stress fibers in one part of the cell (arrows in Fig. 8, *c* and *d*) whereas another part of the cell (arrowheads in Fig. 8, *c* and *d*) showed break up of the stress fiber system and diffuse HSPG labeling. FN was rapidly lost from the cell surface even in cell areas retaining stress fibers (Fig. 8, *e* and *f*). Rounded cells again showed much more cell surface HSPG (Fig. 8*g*) than FN (Fig. 8*h*). Thus, on rounding induced by either EGTA or trypsin, cell surface FN appeared to be rapidly lost, whereas cell surface HSPG was retained even after stress fiber break up.

During the process of cell spreading, the distribution of HSPG was found to correlate closely with that of the actin cytoskeleton whereas that of FN did not. As can be seen in Fig. 9, HSPG (Fig. 9*a*) and FN (Fig. 9*b*) showed different distributions by double immunofluorescence at 1.5 h of spreading. HSPG appeared present on the membrane over the center of unextracted cells and was concentrated in areas of ruffling membrane (Fig. 9*a*). FN appeared generally distributed over the whole cell surface and as fibrillar deposits under the cell center at this stage but showed no concentration in ruffles (Fig. 9*b*). The co-distribution of HSPG, but not FN, with internal actin organization was shown very strikingly at 1 h of spreading (Fig. 9, *c–f*). Both anti-HSPG (Fig. 9*c*) and phalloidin (Fig. 9*d*) staining were concentrated in ruffle areas but FN was absent (Fig. 9, *e* and *f*) from these areas.

DISCUSSION

The anti-HSPG serum used in these studies was raised against cell surface HSPG from rat liver membranes (34, 41). Biochemical analysis shows that it specifically reacts only with HSPG and not with other proteoglycans or proteins. The immunoprecipitation results indicate that the antiserum recognizes HSPG secreted by REF cells into the medium. The reason for immunoprecipitation of only a portion (30%) of the total HSPG is not known, but similar results are obtained when HSPG from hepatocyte medium are tested for reactivity

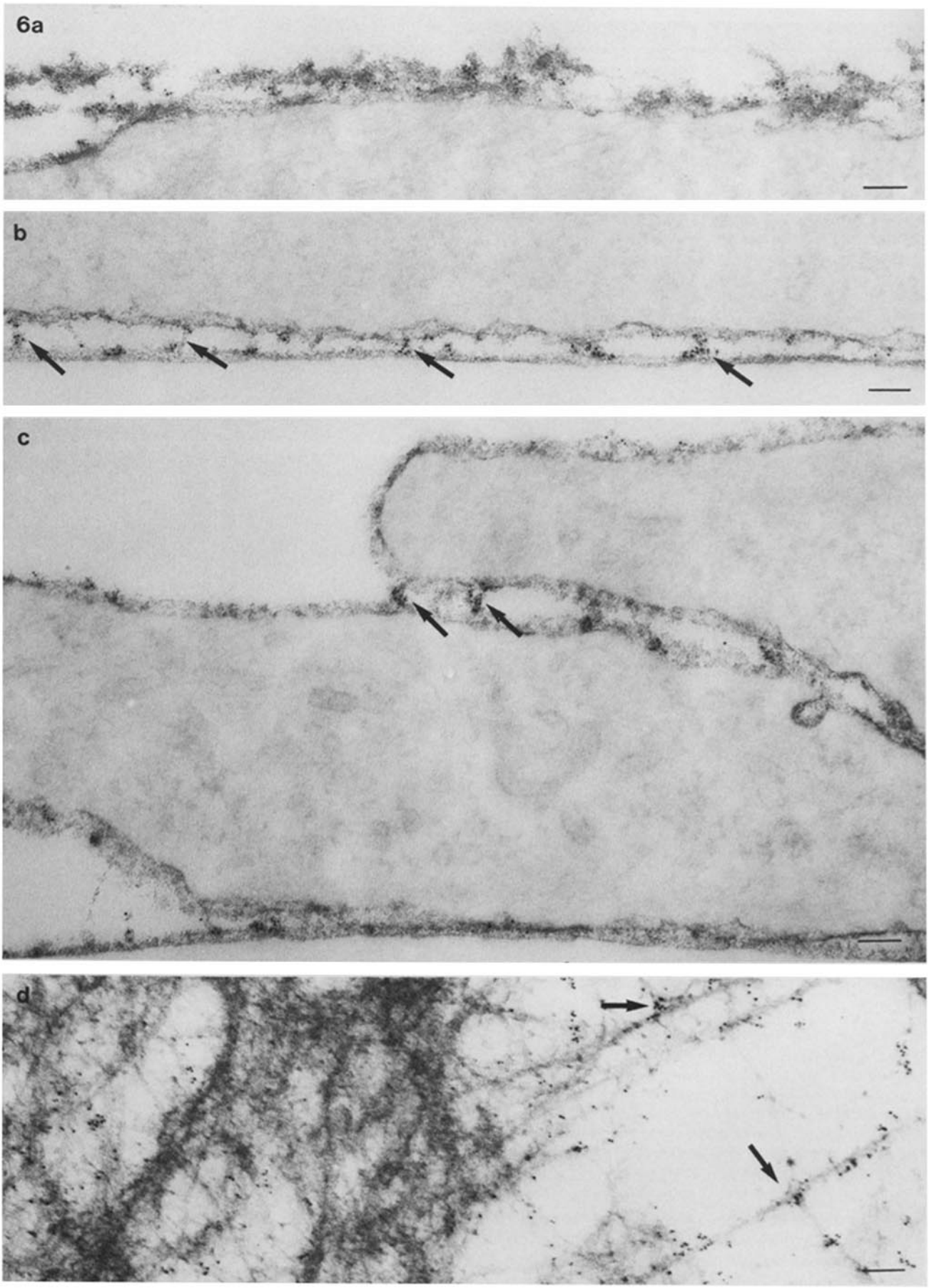

FIGURE 6 Immunoelectron micrographs of REF cells labeled with anti-HSPG followed by colloidal gold anti-rabbit antibodies. (*a*) Dorsal and (*b*) ventral cell surface vertical sections; (*c*) cell-cell contact area, vertical section; and (*d*) horizontal section close to the substrate showing matrix distribution. Arrows denote cell-substratum (*b*) and cell-cell (*c*) contact areas and junctions between fibrils (*d*) labeling for HSPG. Bars, 0.1 μm. × 80,000.

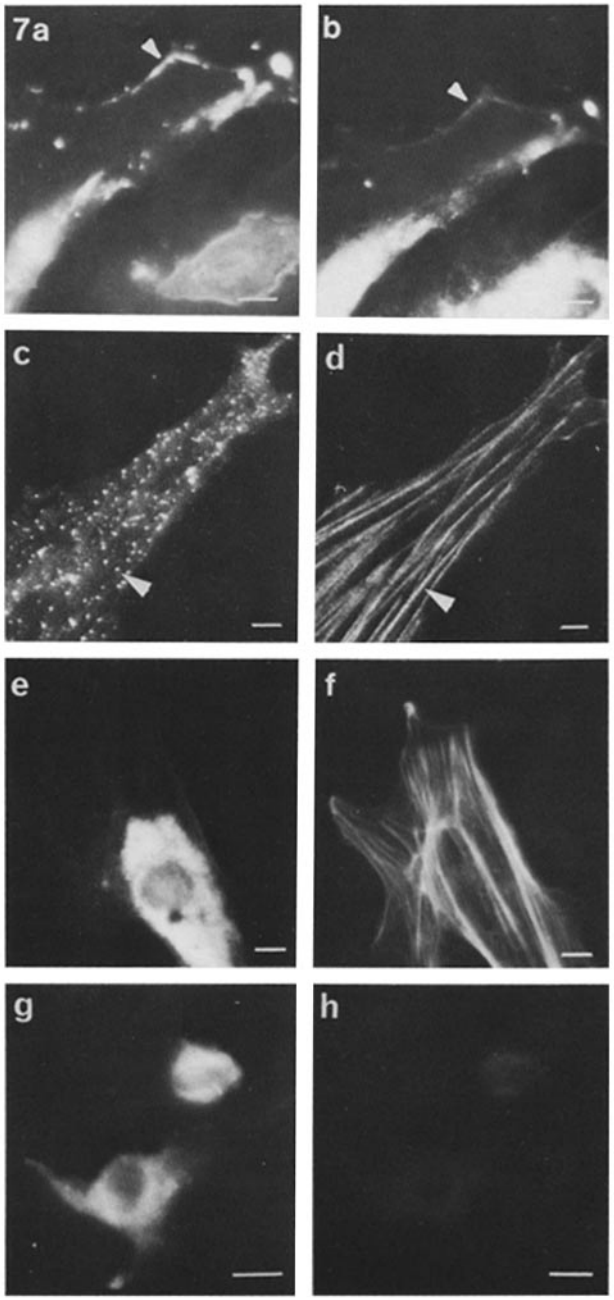

FIGURE 7 Double labeling of REF cells with combinations of anti-HSPG, anti-FN, and phalloidin to monitor HSPG, FN, and actin distribution at stages of rounding induced by trypsin. Pairs of double-labeled micrographs are shown: HSPG (*a*) and corresponding FN (*b*) at 2-min treatment; note areas that label strongly for HSPG but only weakly for FN (arrowheads). HSPG (*c*) and corresponding actin (*d*) at 4-min treatment; arrowheads show HSPG aligning with stress fibers. FN (*e*) and corresponding actin (*f*) at 4 min of treatment. HSPG (*g*) and corresponding FN (*h*) on rounded cells at 6 min of treatment. Cells in *a–f* were permeabilized, whereas *g* and *h* were nonextracted to show only external labeling. Bars, 10 μm. (*a* and *b*) × 520; (*c–f*) × 420; (*g* and *h*) × 630.

against the antiserum, whereas a considerably larger portion of the intercalated cell surface HSPG from rat liver plasma membranes reacts with the antiserum (Kjellén, L., and M. Höök, manuscript in preparation).

Immunofluorescent studies confirm the specificity of the anti-HSPG serum and show that it produces similar staining to that previously seen with an antiserum raised against basement membrane HSPG (24, 26). HSPG is shown here and in previous studies (24, 26) to be present in the extracellular matrix produced by fibroblasts in vitro and to be reduced on transformation. In addition, we show here that matrix deposition of HSPG is preceded by a cell surface distribution.

Anchorage-dependent fibroblasts need to adhere to a suitable substratum and to organize their internal cytoskeleton

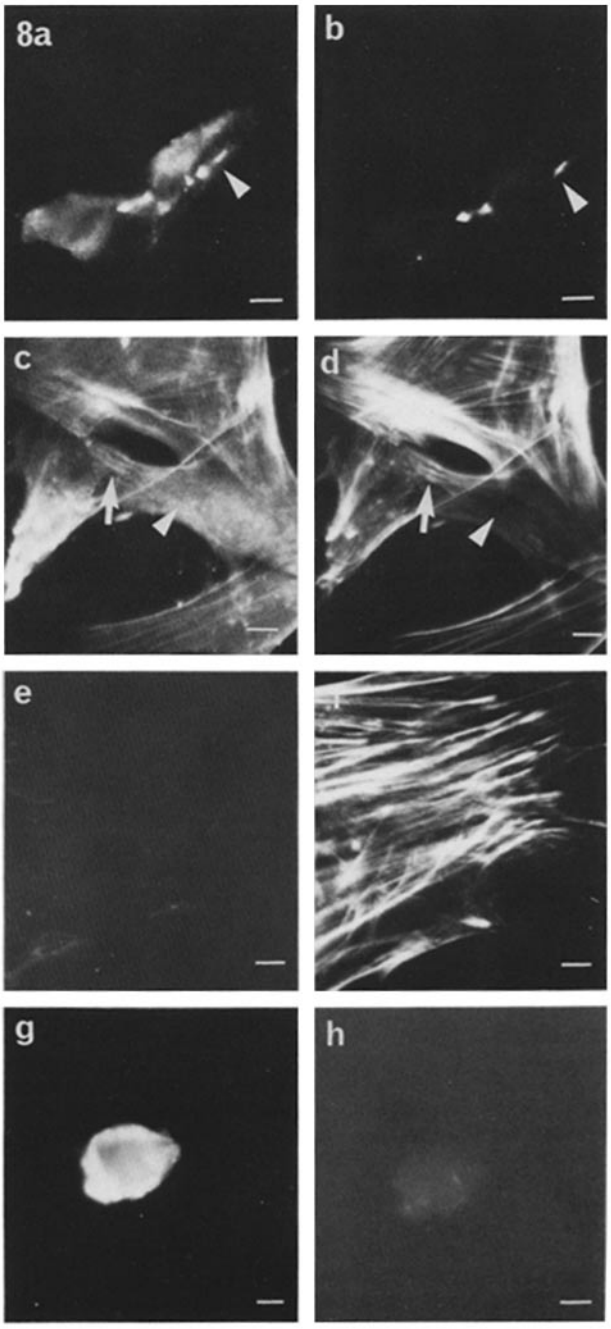

FIGURE 8 Double labeling of REF cells as in Fig. 7 but during EGTA-induced cell rounding. HSPG (*a*) and FN (*b*) at 5 min; note more HSPG than FN (arrowheads). HSPG (*c*) and actin (*d*) at 7 min; note coincidence of linear labeling (arrows) in one part of a cell but diffuse HSPG and actin (arrowheads) in another. FN (*e*) and actin (*f*) at 7 min. HSPG (*g*) and FN (*h*) at 10 min; note retention of more HSPG than FN. *a*, *b*, *g*, and *h* are nonextracted; *c–f* are permeabilized cells. Bars, 10 μm. (*a* and *b*) × 520; (*c–f*) × 420; (*g* and *h*) × 630.

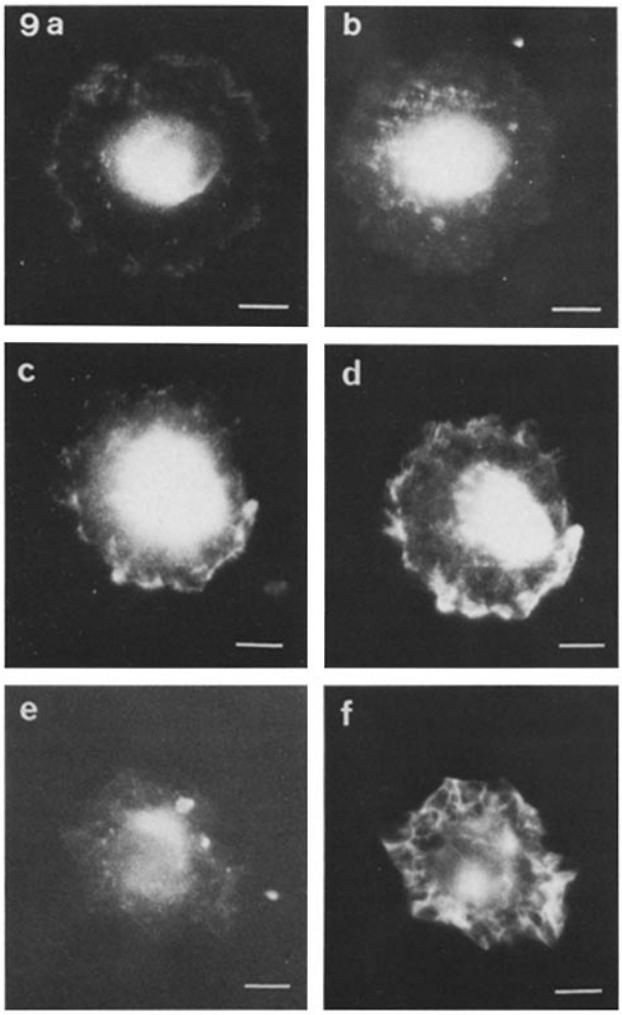

FIGURE 9 HSPG, FN, and actin distributions during spreading. Pairs of micrographs show HSPG (a) and FN (*b*) distribution on nonpermeabilized cells at 1.5 h of spreading; HSPG (c) and actin (*d*) at 1 h of spreading; and FN (e) and actin (*f*) at 1 h of spreading. Bars, 10 μm. × 630.

for survival in vitro. FN has been implicated in this process owing, in part, to the observed co-alignment of FN with stress fibers of normal cells (28, 50) and the promotion of stress fiber formation in transformed cells upon addition of exogenous FN (2, 60). We show here that in sparse normal cells, cell surface HSPG is also aligned as fibrils or as increased membrane concentrations with actin-containing stress fibers which are known to terminate at the ventral cell surface in focal adhesions (1, 6, 25, 29). These are the areas of tight cell-substratum apposition which anchor cells in vitro (1, 25, 29) and have specialized cytoplasmic, membrane, and external molecular and ultrastructural features (3, 8, 22, 36, 38). HSPG at the surface of transformed cells that have no stress fibers visible by phase-contrast microscopy has, however, a diffuse distribution. Both cell types produce an extracellular matrix when cell density and time in culture are increased, although 16C cells deposit less matrix than REF. These matrices contain both HSPG and FN. It is not possible once cell surface matrix fibrils are formed to detect any underlying membrane HSPG concentrations as these may be masked by overlying fibrils.

HSPG and FN are also shown by light microscopy to be present at structures that by interference reflection microscopy are focal adhesions, prepared by mechanical shear of dense cell cultures (3). Previous reports have demonstrated FN labeling of isolated 16C cell focal adhesions together with more strongly labeled remnants of the few matrix fibrils produced by this cell type (3). There is controversy (reference 10 and references cited therein) about the presence of FN at the closest points of cell-substratum attachment, and the limits of resolution at the light microscope level do not permit determination of whether HSPG and FN at focal adhesions are in fact only at the edges of the adhesive plaque. However, since labeling is increased at adhesions with respect to the general membrane staining on intact cells at the high density needed for focal adhesion preparations and also with respect to any surrounding membrane areas remaining loosely attached to isolated adhesions, the present results indicate a concentration of HSPG and FN around these areas. This is in agreement with previous studies (15, 21, 36) showing biochemically that substrate-attached material left behind after EGTA-induced detachment of normal and transformed cells has increased concentrations of HSPG with respect to the rest of the membrane (21). Thus it would appear that although HSPG deposition may be reduced on transformation, the cell-substratum adhesion areas of these cells still have a relative increase in HSPG content.

Immunoelectron microscopy shows HSPG to be localized in discrete areas of the ventral and dorsal surfaces of cells in addition to an extracellular matrix distribution. On cell surfaces, HSPG often appears to be localized at areas of cell-matrix, cell-substratum, and cell-cell contact. On ventral cell surfaces and between overlapping cell margins, discrete areas labeling for HSPG appear to be in association with fibrillar deposits connecting the two surfaces, implicating cell surface HSPG in adhesion processes. In the extracellular matrix, the presence of HSPG at junctions between fibrils may reflect a role for HSPG in matrix formation as previously postulated (12). The close association of FN and HSPG in the matrix is highlighted by problems of antibody accessibility at the light and possibly also the electron microscope level as described in Results, and by the co-purification of FN with HSPG after cesium chloride centrifugation of radiolabeled fibroblast medium (26).

Further evidence for a role of cell surface HSPG in adhesion processes is obtained when the cell surface distributions of HSPG and FN are compared with that of actin during cell rounding and spreading. Previous studies have shown that three separable events occur during cell rounding: loss of fibrillar extracellular matrix FN, break up of an ordered array of membrane glycoproteins which are ricin receptors, and break up of actin stress fibers (6). The present study shows that on trypsination or treatment with EGTA, fibrillar FN is lost from the cell surface before stress fiber break up whereas HSPG labeling is retained longer. On trypsinization, membrane concentrations of HSPG appear to be reduced to linear arrays of particulate staining as stress fibers break up (Fig. 7*c*). This has previously been noted for ricin receptor membrane glycoproteins during cell rounding induced by trypsin (6). This behavior is not seen on EGTA-induced rounding; the linear concentrations of HSPG appear to become more diffuse. It may be that these two agents affect transmembrane interactions in different ways. Even rounded cells retain some diffuse external HSPG membrane staining although this may be reduced. However, no external FN is detectable on rounded

cells, and this argues strongly for a more intimate association of HSPG than of FN with cell membranes. Previous studies (33) have shown that hepatocytes have two forms of cell-associated HSPG, a peripheral form attached to a membrane protein through the glycosaminoglycan chains and a form having its core protein intercalated in the membrane. EGTA would not reduce either population of cell associated HSPG. Trypsin would not remove the glycosaminoglycan chain of peripheral HSPG but could remove portions of the core protein leaving behind peptides attached to the membrane-bound polysaccharide chains. The major portion of the membrane intercalated HSPG could be removed by trypsin; left associated with the membrane would be a part of the core protein containing the hydrophobic anchor. It may well be that the anti-HSPG serum recognizes antigenic determinants on the core protein portion remaining after trypsin treatment. Work is in progress to determine whether REF cells have two types of cell-associated HSPG. Since lung fibroblasts have much less peripherally attached HSPG than hepatocytes (56), we consider that the immunofluorescent staining for HSPG retained on rounded REF cells is mainly due to a membrane intercalated form of HSPG.

It has been postulated (15) that HSPG mediates cell adhesion by bonding between cell surface FN and plasma-derived FN adsorbed onto the substratum, i.e., the bonding being cell-FN-HSPG-FN. Our results monitoring HSPG, FN, and actin distributions during cell rounding, however, argue in addition for a more direct role of HSPG in adhesion, the bonding being cell-HSPG-FN possibly mediated by a transmembrane intercalated form of HSPG. Further evidence for this comes from the cell spreading data that shows that at early stages in spreading, HSPG and actin have similar distributions in ruffles whereas FN is localized differently, apparently in reduced amounts in ruffle areas in comparison with the rest of the cell. Only later does the FN distribution correlate with that of actin (27, 28). Thus in terms of external–internal co-organization in dynamic situations during spreading, HSPG again appears to play a more direct role than FN, possibly mediating the attachment of FN to the cell membrane and coordinating cell surface matrix organization with internal cytoskeleton.

Further evidence for a direct role of HSPG in adhesion processes are the reports that epithelial cells have an intercalated form of HSPG which can bind to actin (45) and that an interaction of cell surface HSPG with a substratum-bound ligand (platelet factor 4) will allow fibroblast adhesion and spreading whereas heparitinase treatment of fibroblasts inhibits its spreading on a FN substrate (37). However, FN-independent spreading using cell surface HSPG is not accompanied by reorganization of the cellular cytoskeleton to give stress fiber formation (37), and it may well be that this requires cell surface HSPG-FN interactions. It should in this context be pointed out that matrix molecules that promote all three stages of fibroblast adhesion (i.e., attachment, spreading, and focal adhesion formation [5, 13]), such as fibronectin (14, 27, 59) and laminin (13), bind sulfated glycosaminoglycans (16, 36, 47, 48, 51). However, the cell binding sites in these adhesive molecules appear to be distinct from those sites binding to polyanionic polysaccharides (44, 53). Thus, although cell attachment and possibly some types of spreading may be promoted by FN or laminin substrates alone, multiple interactions at the cell surface of HSPG and FN or laminin may be needed for focal adhesion formation and cytoskeletal reorganization compatible with growth (4, 14). Further work is in progress to investigate this possibility.

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