The Fibronectin Cell Attachment Sequence Arg-Gly-Asp-Ser Promotes Focal Contact Formation during Early Fibroblast Attachment and Spreading

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Abstract. Cultured fibroblasts form focal contacts (FCs) associated with actin microfilament bundles (MFBs) during attachment and spreading on serum- or fibronectin (FN)-coated substrates. To determine if the minimum cellular adhesion receptor recognition signal Arg-Gly-Asp-Ser (RGDS) is sufficient to promote FC and MFB formation, rat (NRK), hamster (Nil 8), and mouse (Balb/c 3T3) fibroblasts in serum-free media were plated on substrates derivatized with small synthetic peptides containing RGDS. These cultures were studied with interference reflection microscopy to detect FCs, Normarski optics to identify MFBs, and immunofluorescence microscopy to observe endogenous FN fiber formation. By 1 h, 72–78% of the NRK and Nil 8 cells plated on RGDS-containing peptide had focal contacts without accompanying FN fibers, while these fibroblasts lacked FCs on control peptide. This early FC formation was followed by the appearance of coincident MFBs and colinear FN fibers forming fibronexuses at 4 h. NRK and Nil 8 cultures on substrates coated with native FN or 75,000-D FN-cell binding fragment showed similar kinetics of FC and MFB formation. In contrast, the Balb/c 3T3 mouse fibroblasts plated on Gly-Arg-Gly-Asp-Ser peptide-derivatized substrates, or on coverslips coated with 75,000-D FN cell-binding fragment, were defective in FC formation. These results demonstrate that the apparent binding of substrate-linked RGDS sequences to cell surface adhesion receptors is sufficient to promote early focal contact formation followed by the appearance of fibronexuses in some, but not all, fibroblast lines.

FIBRONECTIN (FN)[1] is a large glycoprotein found in plasma and the connective tissue extracellular matrix, which functions primarily in promoting cellular adhesion to solid substrates. It is composed of several protease-resistant functional domains that can bind independently to a number of important substrates, including fibrin, collagen, heparin, and various cell surface components (18, 35). The FN cell-attachment domain has recently been shown to bind to a 140-kD FN receptor complex (1, 2, 33) that co-distributes with microfilament bundles at cell–substrate adhesion sites (4, 5). Through the use of small synthetic peptides deduced from the primary structure of the FN cell-attachment domain, the tetrapeptide Arg-Gly-Asp-Ser has been found to be the minimal FN sequence with cell attachment activity (29–31).

Arg-Gly-Asp-Val, a sequence closely related to Arg-Gly-Asp-Ser, is present in vitronectin, another protein with potent cell-adhesive properties that is localized with FN in the extracellular matrix (15, 16, 44). The vitronectin receptor has a different molecular mass than the FN receptor, and specifically binds vitronectin rather than FN. However, both receptors can recognize Arg-Gly-Asp-containing peptides (32). Therefore, in this report, we shall refer to the cell surface receptors that recognize Arg-Gly-Asp sequences as cell-surface adhesion receptors.

Soon after adhesion to planar substrates coated with either serum or FN, fibroblasts develop several functionally unique types of contact sites at their adhesive surface, as observed with interference reflection microscopy (IRM) (20, 26). Close contacts, which exhibit a substrate separation distance of ~30 nm, are found at the more mobile regions of the cellular attachment surface, and probably represent the weakest type of contact (20). The more circumscribed focal contacts (FCs) possess a membrane–substratum separation gap of only 10 nm, and are apparently a tighter form of adhesion site characteristic of stationary cells. These FCs, which appear black with IRM, are located at the ends of microfilament bundles where these bundles attach to the plasma membrane (20). They are also enriched in the actin-binding protein vinculin (3, 9), and exhibit concentrations of both FN and its 140-kD receptor at their periphery (4–7, 38, 43). A third kind of substrate adhesion site termed the fibronexus (37, 39–42), or extracellular matrix contact site (5, 6), has also been observed in well-spread and stationary fibroblasts.

1. *Abbreviations used in this paper*: DIC, differential interference contrast; FC, focal contact(s); FN, fibronectin; HBSA, heat denatured BSA; IFM, immunofluorescence microscopy; IRM, interference reflection microscopy; SPDP, 3-(2-pyridol-dithio) propionic acid *N*-hydroxy-succinimide ester.

These fibronexus contacts are similar to FCs in that they are circumscribed and elongated, and black when viewed in white light under IRM. The unique features of fibronexus contacts are that they exhibit a co-distribution of FN, 140-kD FN receptor, actin, vinculin, and alpha-actinin throughout the entire contact site, and that they are located along the more centripetal regions of microfilament bundles rather than at their termini. Under electron microscopy, fibronexus contacts are composed of very close tandem or lateral transmembrane associations of 5-nm diameter actin microfilaments and FN fibers (37, 40, 42).

It is evident that heparan sulfate proteoglycans also play a role in FN-mediated fibroblast attachment and spreading (23–27, 34), since they are found at cell–substrate attachment sites (23, 26, 27). Thus it is possible that both the heparin-binding and cell-binding FN domains are involved in fibroblast adhesion, FC formation, microfilament bundle development, and fibronexus morphogenesis. In the series of experiments reported here, we have assessed the ability of the minimum cellular recognition sequence, Arg-Gly-Asp-Ser, to promote these adhesive events during the early phases of fibroblast attachment and spreading on peptide-derivatized substrates. In this system, successful FC formation would presumably occur by peptide binding to the 140-kD FN receptor complex and the vitronectin receptor, but not by attachment to heparan sulfate proteoglycans. Our data suggest that this amino acid sequence alone promotes formation of normal FCs and associated microfilament bundles without FN fibers in hamster and rat fibroblasts, but not in mouse cells.

Materials and Methods

Cells

Normal rat kidney (NRK-49F) fibroblasts and Balb/c 3T3 (clone A31) mouse fibroblasts were obtained from the American Type Culture Collection (Rockville, MD), and Nil 8, a normal hamster fibroblast cell line, was a gift from Dr. Richard O. Hynes (Massachusetts Institute of Technology, Cambridge, MA). They were cultured in Dulbecco's modified Eagle's minimum essential medium (DME) supplemented with 10% fetal bovine serum, and 2 mM glutamine. Cultures were subdivided twice weekly, and discarded after 30 population doublings; cells for these experiments were from passages 11–26. All cell lines used in these experiments were free of *Mycoplasma* contamination as determined using a DNA hybridization assay (Gen-Probe, San Diego, CA).

Proteins and Peptides

Fibronectin was isolated from human plasma obtained from the New York Blood Center (New York, NY) using gelatin–Sepharose affinity chromatography as previously described (36). The 75,000-D fragment of this FN (f75kD) was liberated upon trypsin digestion, and purified by chromatography on columns of DEAE-Sephacel, gelatin–Sepharose, and heparin–Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) as previously described (14). The purity of both FN and f75kD was >99% as judged by SDS PAGE.

To measure the amount of substratum-adsorbed FN, f75kD, and bovine serum albumin (BSA) in cell adhesion experiments, these ligands were radioiodinated with $Na^{125}I$ (New England Nuclear, Boston, MA) using 50 μg/ml chloramine T in 0.05 M phosphate buffer, pH 7.4. After radioiodination, the labeled FN was repurified on gelatin–Sepharose (28), and the ^{125}I-f75kD and ^{125}I-BSA were isolated by chromatography on a Sephadex G-25 (PD-10) column. All samples were dialyzed exhaustively against 0.1 M $NaHCO_3$, pH 8.2, and their final specific activities were 231 Ci/mmole for FN, 176 Ci/mmole for f75kD, and 43 Ci/mmole for BSA. These ^{125}I-radiolabeled molecules were intact as verified by SDS PAGE and autoradiography. Also, the radioiodinated FN and f75kD mediated comparable amounts of cellular attachment relative to unlabeled FN and f75kD in cell adhesion experiments.

Peptides were synthesized on an automated peptide synthesizer (SAM II; Biosearch, Inc., San Rafael, CA) according to the methods of Merrifield. After hydrofluoric acid deprotection, the peptides were purified on a 2.5 × 50-cm HPLC column (ODS-3; Whatman, Inc., Clifton, NJ) using a water–acetonitrile gradient system. The peptides were considered structurally correct by amino acid analysis and proper molecular mass as determined by mass spectroscopy. We synthesized an FN cell-binding peptide, Tyr-Gly-Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro-Cys (peptide 1), and a control peptide with the sequence Tyr-Gly-Arg-Gly-Glu-Ser-Pro-Ala-Ser-Ser-Lys-Pro-Cys (peptide 2). A nonsense tetradecapeptide, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser, was purchased from Peninsula Laboratories, Inc. (Belmont, CA) for use as an alternate control.

Cell Adhesion

We modified the cell adhesion assay of Ruoslahti et al. (36) as follows. Linbro 96-well microtiter polystyrene plates (Flow Laboratories, Inc., McLean, VA) not treated for tissue culture, were coated with 5 μg/ml FN (in 0.1 M $NaHCO_3$, pH 8.2) for 2 h, and washed three times with 0.1 M $NaHCO_3$ using an automated Pro/Pette (Cetus Corp., Emeryville, CA). Alternatively, the wells were coated with heat-denatured BSA (HBSA), derivatized with 3-(2-pyridyl-dithio)propionic acid *N*-hydroxy-succinimide ester (SPDP), a heterobifunctional cross-linking agent (Sigma Chemical Co., St. Louis, MO) to which peptide 1 or 2 was covalently linked via its COOH terminal cysteine (29). 24-well plates (Costar, Cambridge, MA) containing 12-mm diameter glass coverslips treated with 0.1% poly-L-lysine were also coated with FN, f75kD, or with HBSA and derivatized with peptide 1 or 2 as described above, for use in IRM and immunofluorescence microscopy (IFM) experiments. NRK or Nil 8 fibroblasts subcultured 24 h previously, were dispersed using 0.1 mg/ml trypsin (type III S; Sigma Chemical Co.) in Hanks' balanced salt solution (HBSS) containing Ca^{+2} and Mg^{+2} for 3 min, washed three times in DME containing 0.5 mg/ml soybean trypsin inhibitor (Sigma Chemical Co.) and 1 mg/ml HBSA (Sigma Chemical Co.), and resuspended at 1×10^4 cells per Linbro well in DME with 1 mg/ml HBSA.

Trypsin was used to dissociate cells for our adhesion experiments (unless stated otherwise), because FN receptors are resistant to trypsin in the presence of Ca^{+2} (11, 45), and because it also removes residual FN from the cell surface. However, to ensure that trypsin would not be causing significant damage to cell-surface adhesion receptors, the cells for some of our experiments were dissociated with 0.5 mM EGTA as previously described (23).

Competition experiments were also conducted to assess the potency and specificity of peptide adhesion antagonists. In these tests, Linbro microtiter wells were coated with HBSA, and the test peptide cross-linked to the HBSA substrate through its terminal cysteine using SPDP. Serial dilutions of the soluble (nonderivatized) test peptide in DME containing 1 mg/ml HBSA were placed in the titer plate wells, and 1×10^4 cells were seeded per well with the Cetus Pro/Pette. To control for the possibility that the cross-linked HBSA may be degraded by the input cells, which could secrete proteases and adhesive molecules that could secondarily adsorb to the partially denuded substrate, the integrity of peptide-HBSA–coated surface was monitored after seeding using ^{125}I-labeled HBSA. Also, all incubations were performed in the presence of high concentrations of HBSA (e.g., 1 mg/ml) to further coat any putatively exposed substrate adhesion sites. Other controls consisted of uncoated plastic or glass substrates, substrates coated with HBSA and treated with SPDP but not derivatized with peptide, or surfaces to which peptide 2 was covalently linked. For additional controls, peptide 2 or nonsense peptide was added to the media. After a 1-h incubation period at 37°C, any unattached cells were removed by washing with HBSS. Since we found that manual washing by flooding the titer plates with HBSS often caused variable detachment of fibroblasts, we instituted an automated washing protocol using the Cetus Pro/Pette. Each well was mixed three times before medium change with an intermediate pump intensity setting, and three of these cycles were performed on each well. The turbulence generated by this protocol did not detach spread fibroblasts from FN-coated wells, but was sufficient to achieve low backgrounds in uncoated wells, or wells coated with peptide 2 or HBSA. Linbro microtiter cultures established to quantify FN-mediated cell adhesion were fixed in absolute methanol for 30 min and treated with Harris' hematoxylin for 1 h to stain their nuclei, and then air dried. This enabled us to accurately determine the total number of adherent fibroblasts per microtiter well using a customized automated video image analyzer. The video image analyzer apparatus consisted of an image ana-

lyzer (model 3000; Image Technology Corp., Deer Park, NY), a high performance video camera (Vidicon No. 8451; Micron Optics, Inc., Middlesex, NJ) fitted to an autofocus Nikon Diaphot inverted microscope, mounted on a custom built (Image Technology Corp.) optical bench containing a precision motorized microscope stage capable of x and y axis movements with an accurancy ± 5 μm. Each well was subdivided into 35 equal sectors, and the total number of adherent cells determined automatically at a magnification of 195×. The numerical precision was within ±5% of the cell number determined manually.

Microscopy

NRK, Nil 8, or Balb/c 3T3 fibroblasts were plated (2×10^4 cells/1 ml per Costar well) on 12-mm diameter coverslips coated with FN, HBSA, or derivatized with peptide 1 or 2 for correlative IRM, IFM, and differential interference contrast microscopy (DIC) according to the following protocol. Cultures were washed in HBSS at 37°C and fixed in a mixture of 3.5% formaldehyde (freshly generated from paraformaldehyde), 1% glutaraldehyde, and 4.5 mM $CaCl_2$ in 0.1 M Na-cacodylate buffer, pH 7.2, for 30 min at 23°C. After washing in buffer, the fixed cells were permeabilized with 0.1% Triton X-100 in 0.1 M Tris HCl, pH 7.8, for 4 min at 4°C to permit access of antibodies to intracellular FN pools, and to FN fibers that might ordinarily be sealed in the extracellular space beneath the substrate-binding surface. The permeabilized cells were also treated to block possible nonspecific labeling using a modified "blotto" method (22). To do this, coverslips were incubated for 20 min with blotto supernatant (5% wt/vol nonfat dry milk in 0.1 M phosphate buffer, pH 7.8, containing 0.1% NaN_3 and 0.1% BSA centrifuged at 15,000 g for 10 min). The cells were washed with 0.1 M Tris HCl (pH 7.8), and labeled for 1 h with 100 μg/ml monospecific rabbit antifibronectin IgG (42, 43) in 0.1% dry milk, 0.1% BSA, 0.1% NaN_3, and 0.1 M phosphate buffer (pH 7.8) clarified at 15,000 g, followed by 1 h in fluorescein isothiocyanate-conjugated affinity-purified goat anti-rabbit IgG (Boehringer Mannheim, Indianapolis, IN) diluted 1:25 with the same vehicle. These coverslips were mounted on glass slides in a solution of 1.0% n-propyl gallate (12), 1.0% dimethyl sulfoxide, and 0.1% NaN_3 in 0.1 M Tris HCl, pH 7.8, and sealed with clear nail polish. The labeled cells were studied with a Zeiss Photomicroscope III (Carl Zeiss, Inc., Thornwood, NY) fitted with an epifluorescence condenser III RS and an HBO 100 W/2 mercury arc lamp (0.25 × 0.25-mm arc size), using an antiflex neofluor objective lens (63×, 1.25 NA). This instrument was equipped for IRM and DIC as previously described (20). The IRM was performed with either monochromatic light (BP 546/5 interference filter) or with white light so that the various orders of interference colors could be visualized. An epi-insert vertical illuminator with an adjustable diaphragm was used to vary the illuminating numerical aperture, so that the zero order blue-black interference typical of FCs could be distinguished from higher order colors generated by the peripheral cell thickness at low illuminating numerical aperture (20). To further differentiate true FCs from cell thickness artifacts, coverslip cultures were mounted in DME containing 30% BSA (which generated a refractive index of >1.38) as previously described (13). BSA-containing medium caused the disappearance of dark peripheral fringes seen in our cultured cells, while the true FCs similar to those described below were not affected. FN IFM patterns were photographed on Ilford HP-5 film (Microphen developer; 3200 ASA); corresponding IRM and DIC micrographs for each fluorescent field were recorded on Kodak Technical Pan No. 2415 film developed with D-19.

Results

Characterization of FN Cell-binding Peptides

Several groups have shown that peptides with the minimal cell-surface receptor recognition signal Arg-Gly-Asp-Ser interact with FN or vitronectin receptors (1, 2, 29–33). Since peptide 1 (Tyr-Gly-Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro-Cys) contains this sequence, solutions with sufficiently high concentrations of functional peptide 1 should inhibit cellular attachment to FN, or to peptide 1–derivatized substrates. Furthermore, cells should attach directly to substrates derivatized with peptide 1 under serum-free conditions. We performed experiments to assess the functionality of peptide 1 under these circumstances. When attachment of

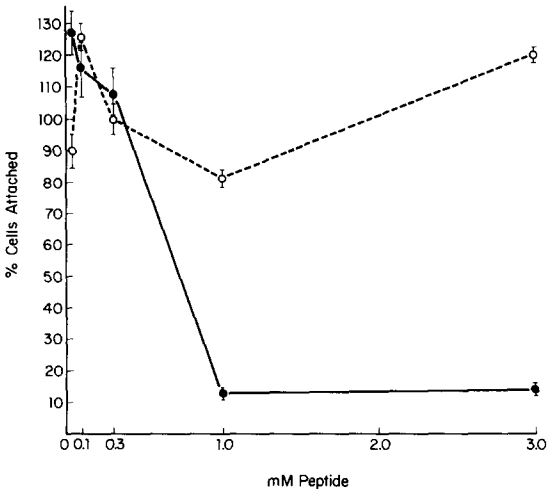


Figure 1. Inhibition of fibroblast attachment to substrates coated with FN by Gly-Arg-Gly-Asp-Ser-containing peptide 1. Suspensions of 1×10^4 NRK cells in serum-free medium with 1 mg/ml HBSA, and various concentrations of peptide 1 (*solid circles*), or control peptide Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser (angiotensin I precursor) (*open circles*), were added to Linbro microtiter wells precoated with 5 μg/ml FN. After 1 h, nonadherent cells were removed with a standard washing force, and the attached cells fixed, stained, and counted as described in Materials and Methods. Each point represents the mean percentage (relative to untreated cells on FN) of attached cells per well (performed in triplicate), and the brackets are plus or minus one standard error.

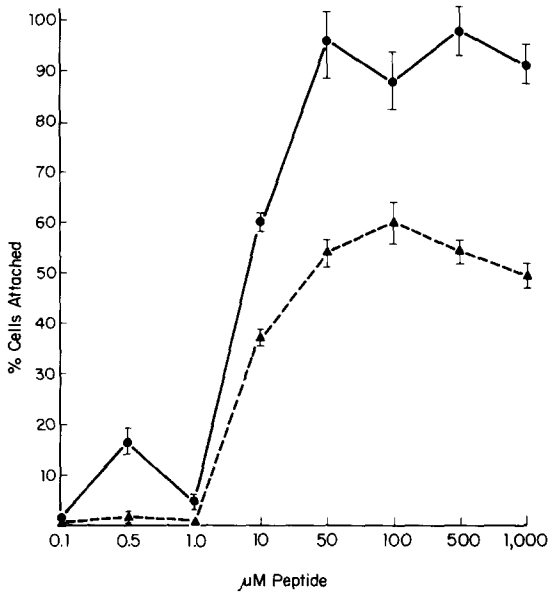


Figure 2. Adhesion of NRK fibroblasts (*circles*) or Nil 8 cells (*triangles*) to substrates derivatized with Gly-Arg-Gly-Asp-Ser-containing peptide 1. Suspensions of 1×10^4 cells in serum-free medium with 1 mg/ml HBSA were seeded into wells cross-linked with various dilutions of peptide 1. After a 1-h incubation period, we performed automated washing, fixation, staining, and counting as detailed in Materials and Methods. The experiment was performed in triplicate, and the points represent the means of the percentage adherent cells per well; the bars indicate plus or minus one standard error. Background levels of ≤13% attached cells per well were observed in wells derivatized with control peptide 2 containing the Gly-Arg-Gly-Glu-Ser sequence (data not shown).

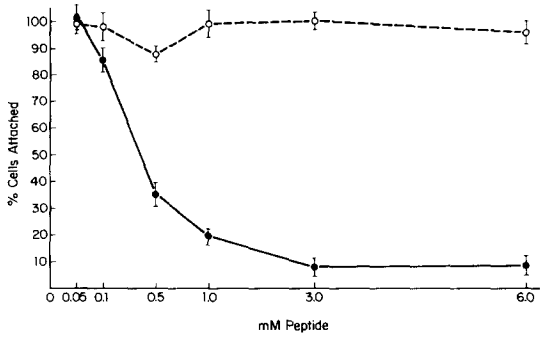


Figure 3. Inhibition of NRK fibroblast attachment to polystyrene microtiter wells derivatized with Gly-Arg-Gly-Asp-Ser–containing peptide 1 by various concentrations of soluble peptide 1 in serum-free medium. Suspensions of 1 × 10^4 cells were dispersed into wells whose surfaces were cross-linked with peptide 1 (previously applied from a 50-μM solution). The culture media contained 1 mg/ml HBSA and various concentrations of soluble FN peptide 1 (*solid circles*), or control peptide 2 (with the nonbinding sequence Gly-Arg-Gly-Glu-Ser) (*open circles*). After the 1-h attachment period, the cultures were washed, processed, and counted as described in Materials and Methods. Each point represents the mean of six separate determinations of the percentage of adherent cells per well. The 100% adhesion value was obtained by counting the number of cells attached to peptide 1–coated wells that were incubated and washed with media without soluble peptide.

NRK cells to FN-coated plastic was challenged with millimolar concentrations of peptide 1 dissolved in culture medium containing 1 mg/ml HBSA, adhesion was inhibited by 90%, while only low levels of inhibition were observed with a control tetradecapeptide (Fig. 1). We then determined the quantity of peptide 1 (in solution) to be cross-linked to SPDP-derivitized substrates required to promote attachment of NRK or Nil 8 fibroblasts in serum-free media. In additional radiolabeling experiments (see below), we measured the relative amounts of peptide 1 and FN bound to the substratum, and the stability of substrate-linked BSA-peptide complexes during cell attachment and FC formation. Both cell types exhibited maximal attachment when a solution of 50 μM (or higher) concentration of FN peptide 1 was covalently linked to the substrate (Fig. 2). For a control, we also cross-linked peptide 2 to the substrate. Peptide 2 (Tyr-Gly-Arg-Gly-Glu-Ser-Pro-Ala-Ser-Ser-Lys-Pro-Cys) has the modified sequence Gly-Arg-Gly-Glu-Ser, which apparently does not bind to substrate adhesion receptors (31). As an additional control, cells were seeded onto SPDP-derivatized BSA without peptide. Both control substrata did not promote cell attachment above background levels (not shown). In a final experiment, substrates were derivatized with a 50-μM solution of peptide 1, and the ability of NRK fibroblasts to attach to these surfaces in media containing soluble peptide 1, or control peptide 2, was determined (Fig. 3). Culture media

*Table I. Percentage of Cells with FCs after Seeding on Gly-Arg-Gly-Asp-Ser Tridecapeptide, or FN-coated Substrates**

Cell line	Substrate	Time after seeding 1 h	2 h	4 h
NRK	Peptide 1	72	69	95
NRK	FN	68	82	85
Nil 8	Peptide 1	78	73	90
Nil 8	FN	72	90	95

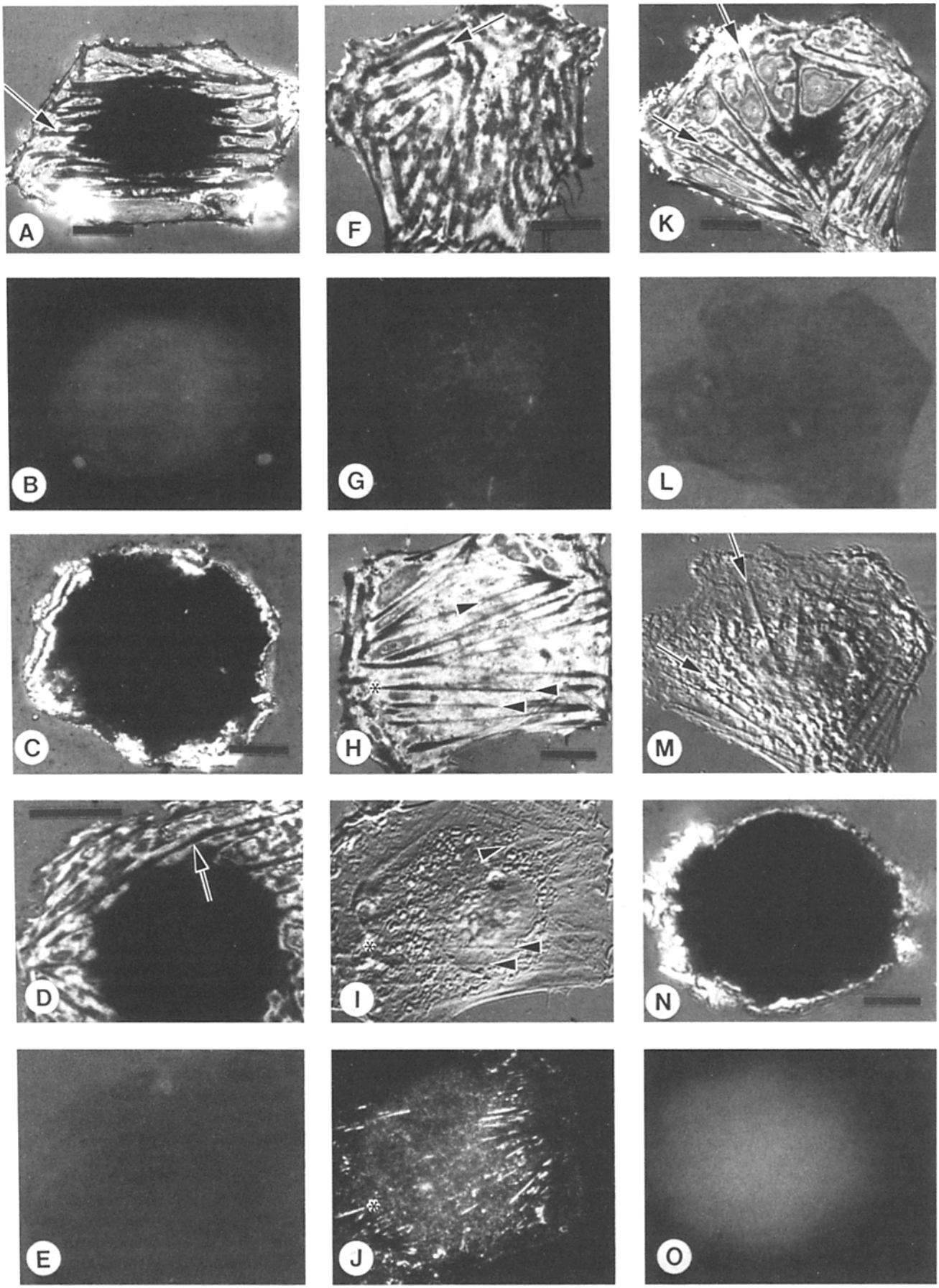
* Cells harvested with trypsin and suspended in serum-free DME containing 1 mg/ml HBSA were seeded at 2 × 10^4 cells/ml per well onto 12-mm diameter glass coverslips derivatized with Gly-Arg-Gly-Asp-Ser tridecapeptide or coated with FN (5 μg/ml) as described in Materials and Methods. Only those cells containing 10 or more FCs were scored positively. Percentages represent the means from two experiments in which 100 cells were sampled per trial. In each case, the standard error was ≤7.3% of the mean.

*Table II. FC Formation in Fibroblasts Cultured on Substrates Coated with FN, 75,000-D FN Cell-binding Fragment, or Gly-Arg-Gly-Asp-Ser Tridecapeptide**

Cell line	Substrate	Time after seeding 30 min	2 h	6 h
NRK	FN	4	95	60
NRK	f75kD	5	85	62
NRK	Peptide 1	2	62	48
Nil 8	FN	3	100	100
Nil 8	f75kD	3	91	–
Nil 8	Peptide 1	0	75	69
Balb/c 3T3	FN	1	40	37
Balb/c 3T3	f75kD	0	15	10
Balb/c 3T3	Peptide 1	0	4	4

* Fibroblasts were detached with EGTA, resuspended in serum-free DME containing 1 mg/ml HBSA and plated at 2 × 10^4 cells/ml per well onto 12-mm diameter coverglasses coated with FN (5 μg/ml), 75,000-D FN cell-binding fragment (5 μg/ml), or derivatized with Gly-Arg-Gly-Asp-Ser tridecapeptide as described in Materials and Methods. Data are expressed as the mean percentage of cells with FCs per 100 cells counted. Two coverslips were sampled for each value and the standard errors were ≤6.7%.

Figure 4. FC and microfilament bundle formation in rat (NRK) fibroblasts at early culture times (1–2 h) on coverglasses derivatized with Gly-Arg-Gly-Asp-Ser–containing peptide 1 or FN. Cells (2 × 10^4/well) were seeded in serum-free media, fixed, permeabilized, indirectly immunostained for FN, and photographed with IRM, IFM, and DIC as described in Materials and Methods. All internal standards equal 10 μm. (*A*) Several focal contacts (*arrow*) are seen with IRM in this spreading NRK cell seeded on a peptide 1 substrate 1 h before fixation. The central dark spot is due to higher order interference produced by the thicker portions of this cell. (*B*) IFM of cell in *A*. FN fibers are not detectable in this cell. (*C*) IRM of typical rounded cell that totally lacks FCs after 1-h culture on Arg-Gly-Glu-Ser–containing peptide 2. (*D*) IRM of NRK cell plated on FN-coated glass for 1 h exhibits FCs (*arrow*) similar to those formed on peptide 1. (*E*) Field in *D* viewed with IFM shows diffuse FN staining of the substrate (*upper left*), but no FN fibers beneath the cell. (*F*) Spread cell in 2-h culture on peptide 1 has prominent focal contacts (*arrow*) seen with IRM, but lacks FN fibers under IFM (*G*). *H*, *I*, and *J* is a series typifying the minority of the 2-h cells seeded on peptide 1 that have prominent microfilament bundles and FN fibers. (*H*) Under IRM, these cells have tight FCs (*asterisk*) that merge with slightly weaker substrate adhesions (*arrowheads*) coincident with the microfilament bundles seen under DIC (*arrowheads* in *I*). (*J*) FN fibers observed with IFM at the substrate surface are mostly unrelated to these contacts, except for one FN fiber (*asterisk*) that is tandemly associated with an FC (see corresponding asterisks in *H* and *J*). The series *K*, *L*, and *M* shows an NRK fibroblast spread on an FN substrate for 2 h. Corresponding pairs of arrows depict FCs (*K*; IRM) coincident with microfilament bundles under DIC (*M*). (*L*) No FN fibers are seen beneath this cell using IFM. (*N*) IRM of 2-h control culture where the cells were plated on peptide 2; no FCs are seen. (*O*) IFM of cell in *N* labeled for FN; FN fibers are absent.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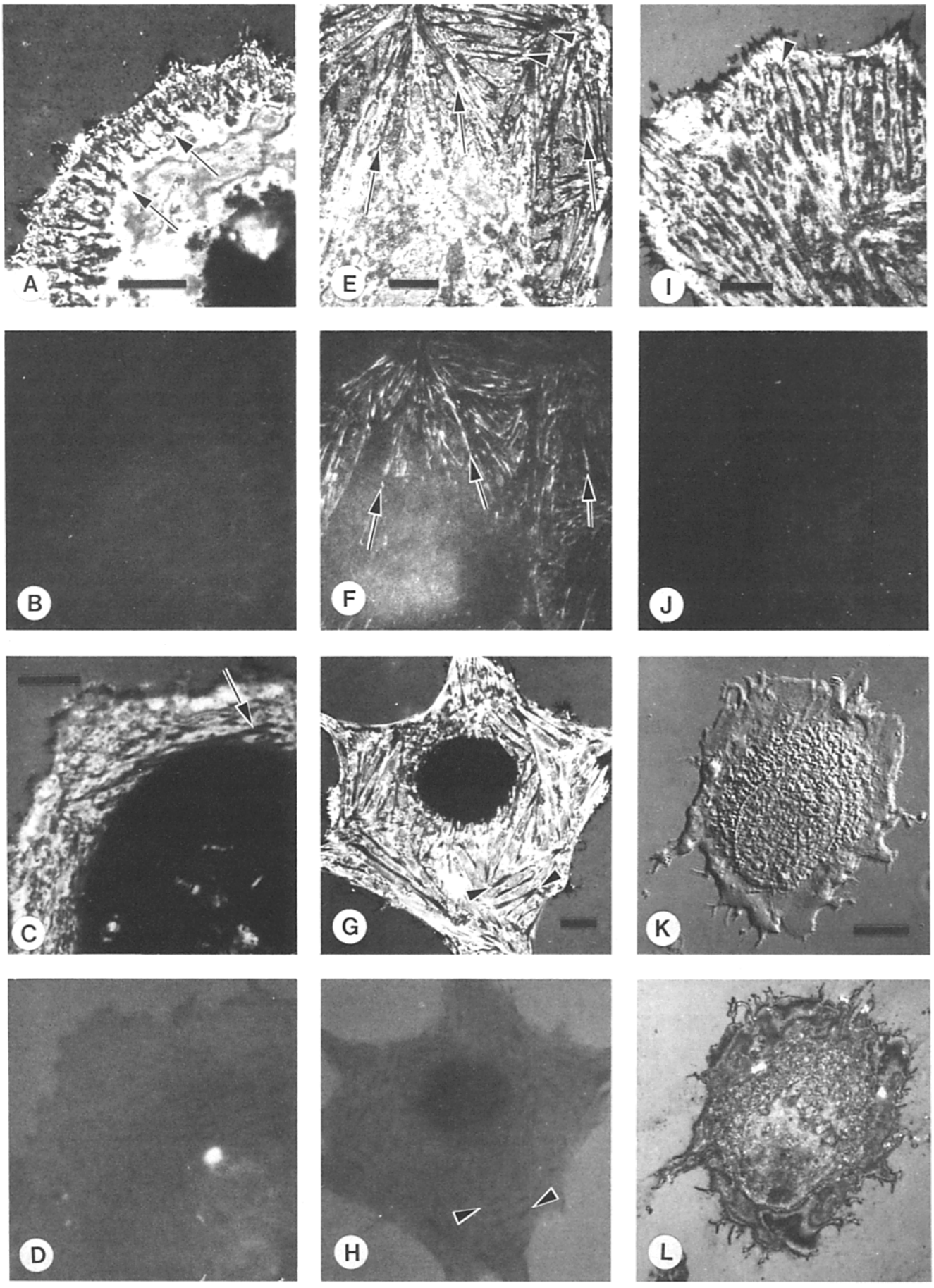
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with millimolar concentrations of Gly-Arg-Gly-Asp-Ser-containing peptide 1 inhibited cellular adhesion to solid-phase-linked peptide 1 by >90%, while the control peptide 2 merely produced ~10–15% inhibition. Based on this competition experiment, we conclude that peptide 1 is capable of promoting fibroblast adhesion through the interaction of its Gly-Arg-Gly-Asp-Ser sequence with cell-surface adhesion receptors.

Development of FCs, Microfilament Bundles, and FN Fibers

After establishing that substrates derivatized with our Gly-Arg-Gly-Asp-Ser-containing tridecapeptide 1 promoted adhesion of fibroblasts, we studied these cultures with IRM to determine if FC formation was occurring. As shown in Tables I and II, few FCs were seen before 1 h, while 69–95% of the NRK and Nil 8 fibroblasts seeded onto surfaces derivatized with peptide 1 formed FCs 1–4 h after seeding. This percentage was similar to the number of cells with focal contacts observed on FN-coated substrates. The method of cell dissociation (i.e., trypsin vs. EGTA) had no apparent effect on the kinetics of FC formation. The earliest FCs observed at 1 h on peptide 1–coated substrates were not associated with FN fibers at the adhesive cell surface (Fig. 4, *A* and *B*; Fig. 5, *A* and *B*); microfilament bundles were not seen at this time (data not shown). No FCs were found on the few rounded cells that attached to control substrates derivatized with Gly-Arg-Gly-Glu-Ser-containing peptide 2 (Fig. 4 *C*). Fibroblasts plated onto FN-coated coverslips had morphologically similar FCs without FN fibers at the 1-h interval (Fig. 4, *D* and *E*; Fig. 5, *C* and *D*). By 2 h in culture, the majority of the cells that had formed FCs on Gly-Arg-Gly-Asp-Ser peptide 1 substrates still lacked FN fibers (Fig. 4, *F* and *G*; Fig. 7). However, ~30% of these fibroblasts had FN fibers (Fig. 4, *H–J*; Fig. 5, *E* and *F*; Fig. 7), which were usually not coincident with FCs. These FN-positive cells also exhibited microfilament bundles at their adhesive surfaces that were coincident with FCs, but they were not usually aligned with FN fibers (Fig. 4 *I*). Interestingly, cells cultured for 2 h on FN-coated coverslips also showed well-formed FCs aligned with prominent microfilament bundles, but they completely lacked FN fibers (Fig. 4, *K–M*; Fig. 5, *G* and *H*), although the substrate was diffusely FN-positive. Similar cells plated on FN substrates but incubated with preimmune IgG as controls showed no FN immunofluorescence (Fig. 5, *I* and *J*). Low quantities of cells attached to control Gly-Arg-Gly-Glu-Ser-containing peptide 2 substrates (Fig. 4, *N* and *O*), or to BSA-coated substrates treated with SPDP (Fig. 5, *K* and *L*), and were not spread. These rounded cells completely lacked FCs, FN fibers, and microfilament bundles.

We observed the striking development of substrate adhesion complexes, consisting of colinear focal contacts, FN fibers, and microfilament bundles at later culture times (4–8 h) in cells on substrates coated with peptide 1 in serum-free media (Fig. 6). Many of these intensely immunostained FN fibers were precisely coincident with microfilament bundles and FCs at the substrate-binding surface (see corresponding pairs of arrowheads in Fig. 6, *A–F, J* and *K*). The close coincidence of these adhesive structures was assessed by overlapping transparencies of the micrographs as previously described (43). These closely apposed FN fibers and actin microfilaments localized at FCs are probably fibronexuses, or extracellular matrix adhesion sites (6), attached to the substrate. Again, companion cultures established on FN-coated coverslips had well-developed FCs coincident with microfilament bundles, but lacked conspicuous substrate-associated FN fibers (Fig. 6, *G–I*). The few fibroblasts attached to coverslips coated with BSA or control peptide 2 were not well spread, did not have FCs, and lacked substrate adhesion complexes (Fig. 6 *L*).

The quantity of NRK or Nil 8 fibroblasts with substrate-associated FN fibers was also determined as a function of culture time and substrate composition. Cells plated onto coverglasses derivatized with either Gly-Arg-Gly-Asp-Ser-containing peptide 1, FN, or BSA, were scored for the presence of FN fibers with IFM (Fig. 7). The percentage of FN fiber-positive cells varied from 20–40% during culture times of 1–2 h on peptide 1 substrates. Most of the cells in these early cultures lacked FN fibers, and there was little relationship between the distributions of FCs and FN fibers in the FN-positive cells. However, by 4 h or beyond, the majority of fibroblasts cultured on glass derivatized with peptide 1 had FN fibers (Fig. 7) coincident with FCs, and actin microfilaments in substrate adhesion complexes (Fig. 6, *A–F*). Surprisingly, fibroblasts seeded onto FN-coated coverglasses failed to develop FN fibers until longer culture periods (8–10 h) (Fig. 7); these FN fibers were also coaligned with FCs and microfilaments.

FC Formation in Mouse Fibroblasts on Gly-Arg-Gly-Asp-Ser Peptide, f75kD, or FN-coated Substrates

After completion of the above experiments in NRK (rat) and Nil 8 (hamster) fibroblasts, other studies appeared with apparently contradictory results (19, 21, 46). These reports concluded that Balb/c 3T3 (mouse) fibroblasts and human embryonic fibroblasts were not capable of forming FCs on

Figure 5. Morphogenesis of FCs in 1–2-h cultures of hamster (Nil 8) fibroblasts seeded on substrates coated with peptide 1 or FN. Culture and preparation conditions are indicated in the legend to Fig. 4; the internal scales are 10 μm in length. Cells in 1-h cultures on peptide 1 exhibited FCs (*arrows* in *A*; IRM), but had no FN fibers detectable with IFM (*B*). Analogous 1-h cultures on FN-coated substrates showed similar FCs (*arrow* in *C*; IRM) without immunofluorescent FN fibers (*D*). By 2-h the Nil 8 fibroblasts plated on peptide 1 developed striking focal contacts (*E*; IRM), and a minority of these cells also had FN fibers (*F*; IFM). Some of the FCs were not coincident with FN fibers (*arrowheads* in *E*) while other FCs co-distributed with FN fibers (corresponding *arrows* in *E* and *F*). Similar focal contacts were seen with IRM in the Nil 8 cells cultured on FN substrates for 2 h (*arrowheads* in *G* and *I*). Although IFM of these preparations showed a uniform distribution of FN on the substrate (*H*), FN staining was depleted at FCs (*arrowheads*) and no FN fibers were observed at the substrate. *I* and *J* show a control group in which companion FN-coated coverslips with FC-rich cells (*I*; IRM) were unstained (*J*) when FN antibodies were replaced by preimmune IgG in our IFM protocol. *K* and *L* depict poorly spread cells from 2-h cultures on SPDP-treated BSA without peptide. These cells lacked microfilament bundles under DIC (*K*) and did not contain FCs under IRM (*L*).

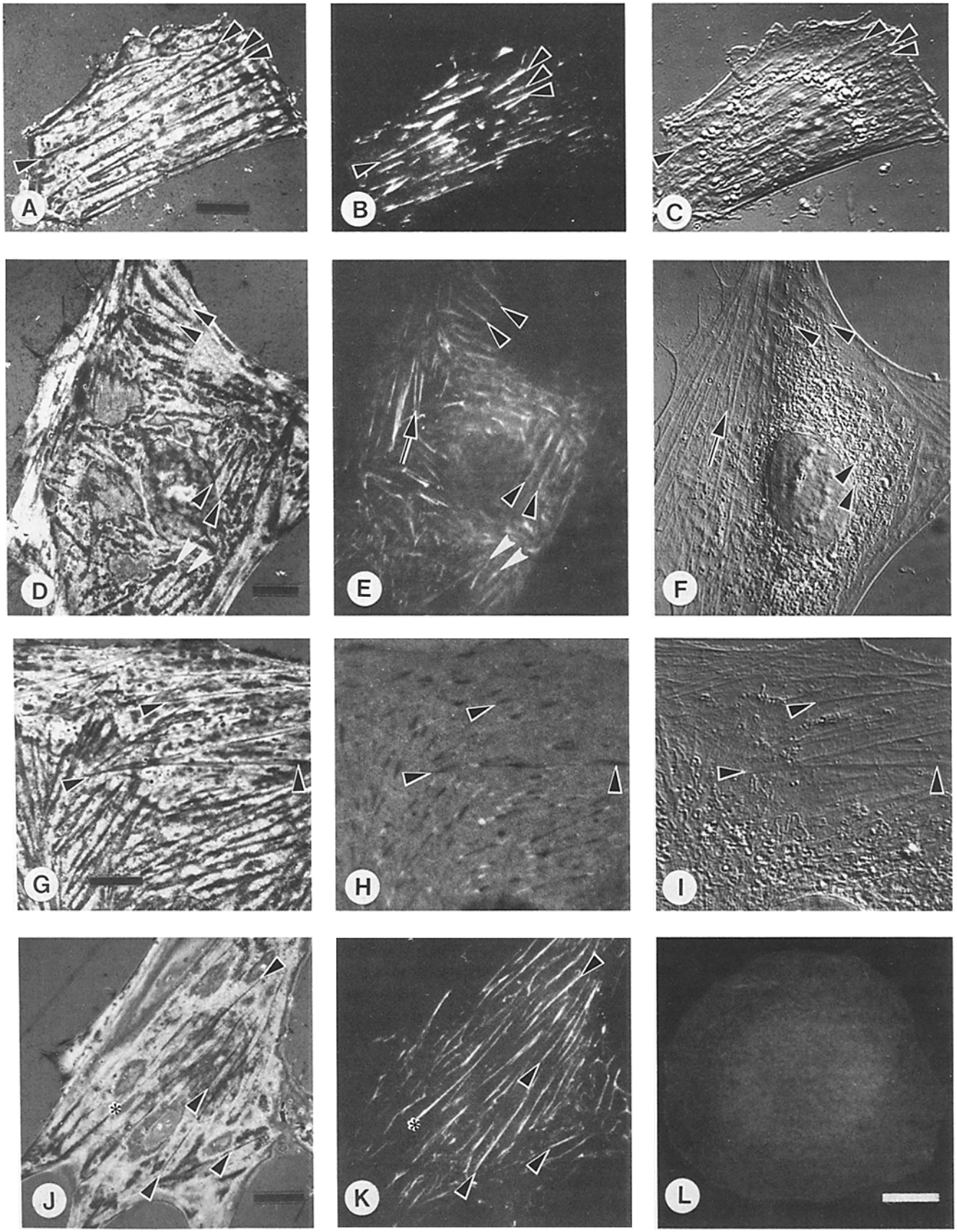

Figure 6. Appearance of substrate adhesion complexes (fibronexuses) composed of colinear FCs, microfilament bundles, and FN fibers in later cultures (4–8 h) of NRK or Nil 8 fibroblasts seeded onto coverglasses derivatized with Gly-Arg-Gly-Asp-Ser–containing peptide 1 as described in the legend to Fig. 4. Correlative micrographs were taken with IRM to see FCs (*A*, *D*, *G*, and *J*), with IFM to visualize FN fibers (*B*, *E*, *H*, and *K*), and with DIC to resolve actin microfilament bundles (*C*, *F*, *I*). Internal standards represent 10 μm. *A*, *B*,

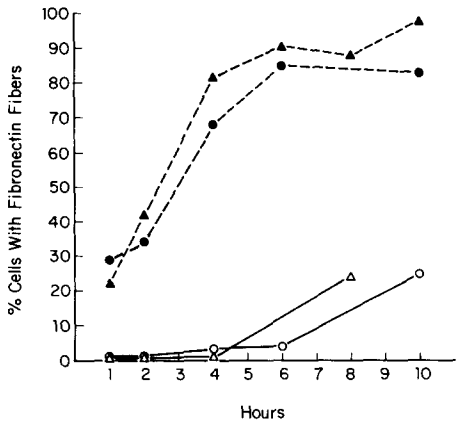

Figure 7. Time course of the appearance of fibroblasts with substrate-attached FN fibers. NRK cells (*triangles*) or Nil 8 fibroblasts (*circles*) were plated (2×10^4 cells/well) on 12-mm diameter coverglasses derivatized with Gly-Arg-Gly-Asp-Ser-containing peptide 1 (*solid symbols, broken lines*), or coated with FN (*open symbols, solid lines*), and allowed to attach in serum-free medium containing 1 mg/ml HBSA. After washing, fixation, permeabilization, and immunofluorescent staining with FN antibodies, the cultures were scored for FN fibers with IFM. NRK cells seeded on control substrates composed of BSA-coated coverglasses treated with SPDP did not develop FN fibers, and BSA control Nil 8 cultures also lacked FN fibers at all culture times except 10 h, where 13% of the cells were FN-positive (control data omitted). Each point represents the mean percentage of two experiments, in which a minimum of 100 cells were scored per coverslip; the standard errors were ≤4.5% of each mean value.

substrates coated with the FN cell-binding domain containing the Arg-Gly-Asp-Ser cell-adhesion sequence. Therefore, we conducted additional experiments to assess FC formation in Balb/c 3T3, NRK, and Nil 8 fibroblasts seeded in serum-free media onto substrates coated with either Gly-Arg-Gly-Asp-Ser peptide 1, FN, or the f75kD FN cell-binding domain. We found that the Balb/c 3T3 mouse fibroblasts were not capable of forming significant quantities of FCs on substrates coated with either f75kD or peptide 1 (Fig. 8, *E* and *F*), while the rat and hamster cells formed numerous FCs on these molecules (Fig. 8, *B*, *C*, *H*, and *I*; Table II). In addition, the mouse fibroblasts formed substantially lower numbers of FCs on FN substrates, relative to the NRK and Nil 8 lines (Table II). These results suggest that the above contradictions are due to cell-type differences in ability to engage in FN-mediated FC formation.

Measurement of Substrate-bound Gly-Arg-Gly-Asp-Ser Peptide, f75kD, and FN

To compare the capacities of substrates coated with peptide 1, f75kD, or FN to promote focal contact formation, we determined the molar quantities of these ligands bound to surfaces used for cell attachment. As shown in Table III, the number of moles of peptide 1 bound per square micrometer substrate greatly exceeded the relative amounts of either FN or f75kD attached. We also determined that (1.77×10^{-20}) moles of BSA were cross-linked per square micrometer of coverslip surface, which compared favorably with the molar amounts of f75kD and FN adsorbed. These data indicate that 425 mol of peptide 1 were cross-linked to each mole of BSA adsorbed to the coverslip, thus providing a substratum with a 1,651-fold enrichment of Gly-Arg-Gly-Asp-Ser sites relative to the coverslips coated with FN or f75kD.

*Table III. Substrate Adsorption of Fibronectin Ligands**

Ligand	Concentration	Coverslip surface	Relative number RGDS sites
		moles bound/μm²	
FN	11.4 nM	2.28×10^{-21}	—§
f75kD	66.7 nM	8.93×10^{-21}	1.95
Peptide 1‡	50.0 μM	7.53×10^{-18}	1,651

* Measured on 12-mm diameter poly-L-lysine-treated coverslips used to study FC formation. Coverslips were incubated in solutions of radioiodinated FN and f75kD as described in Materials and Methods, and the amount of ligand bound per coverslip determined with a gamma counter.
‡ Quantities of peptide 1 cross-linked to 12-mm diameter coverslips containing 2.0×10^{-12} mol of bound BSA (linked with SPDP as described in Materials and Methods) were calculated by determining the loss of monomeric peptide from solution using high pressure liquid chromatography. The *P* value of this difference was <0.05 as determined with Student's paired *t* test.
§ The quantities of Arg-Gly-Asp-Ser sites bound per square micrometer were determined relative to FN, based on the presence of two Arg-Gly-Asp-Ser sequences in each FN molecule adsorbed.

We also were concerned about the possibility that putative cell-derived proteases might degrade the peptide-BSA complexes cross-linked to the substrate, thus rendering our calculations invalid. To check this possibility, coverslips were adsorbed with ^{125}I-labeled BSA and peptide 1 was cross-linked to these BSA-coated surfaces using SPDP. We then incubated these peptide-derivatized coverslips with either 2×10^4 NRK fibroblasts per milliliter of serum-free medium containing 1 mg/ml HBSA, or with medium alone, for 1, 2, 4, and 6 h at 37°C. We found that the presence of attached cells correlated with no significant (0–1.6%) release of labeled BSA into the medium (data not shown). Since relatively large quantities of peptide-BSA complexes were cross-linked to the substratum for our experiments, the negligible release of these complexes probably had little influence on the FC formation that occurred during this time period.

Discussion

Our results show that two different fibroblast lines (normal

and *C* depict an NRK fibroblast fixed 6 h after seeding onto peptide 1. Tight linear FCs (*arrowheads*) seen with IRM in *A* are coincident with immunostained FN fibers in *B* (corresponding *arrowheads*; IFM), and actin microfilament bundles in *C* (matching *arrowheads*; DIC). In *D*, *E*, and *F*, a Nil 8 cell on peptide 1 is shown after 4 h in culture. Pairs of corresponding black arrowheads depict FCs in *D* coincident with FN fibers in *E*, and actin microfilament bundles in *F*. The white arrowheads show co-distributions of FCs in *D* and FN in *E* without detectable microfilament bundles. The corresponding arrows in *E* and *F* depict FN fibers coincident with microfilament bundles that lack observable FCs. *G*, *H*, and *I* illustrate another Nil 8 fibroblast cultured on an FN-coated substrate for 4 h. Arrowheads indicate coincident associations of FCs in *G*, microfilament bundles in *I*, and discontinuities in the FN coating in *H*. A few small FN fibers are tandemly associated with these discontinuities, but prominent FN fibers are absent (*H*). *J* and *K* show a Nil 8 cell prepared 8 h after plating on a peptide 1–derivatized substrate. Arrowheads depict several linear FCs in *J* coaligned with FN fibers in *K*; the asterisks show an FC and FN fiber in tandem association. (*L*) An IFM photomicrograph of a rounded Nil 8 fibroblast cultured on a peptide-lacking SPDP-treated BSA substrate for 8 h; FN fibers are not present.

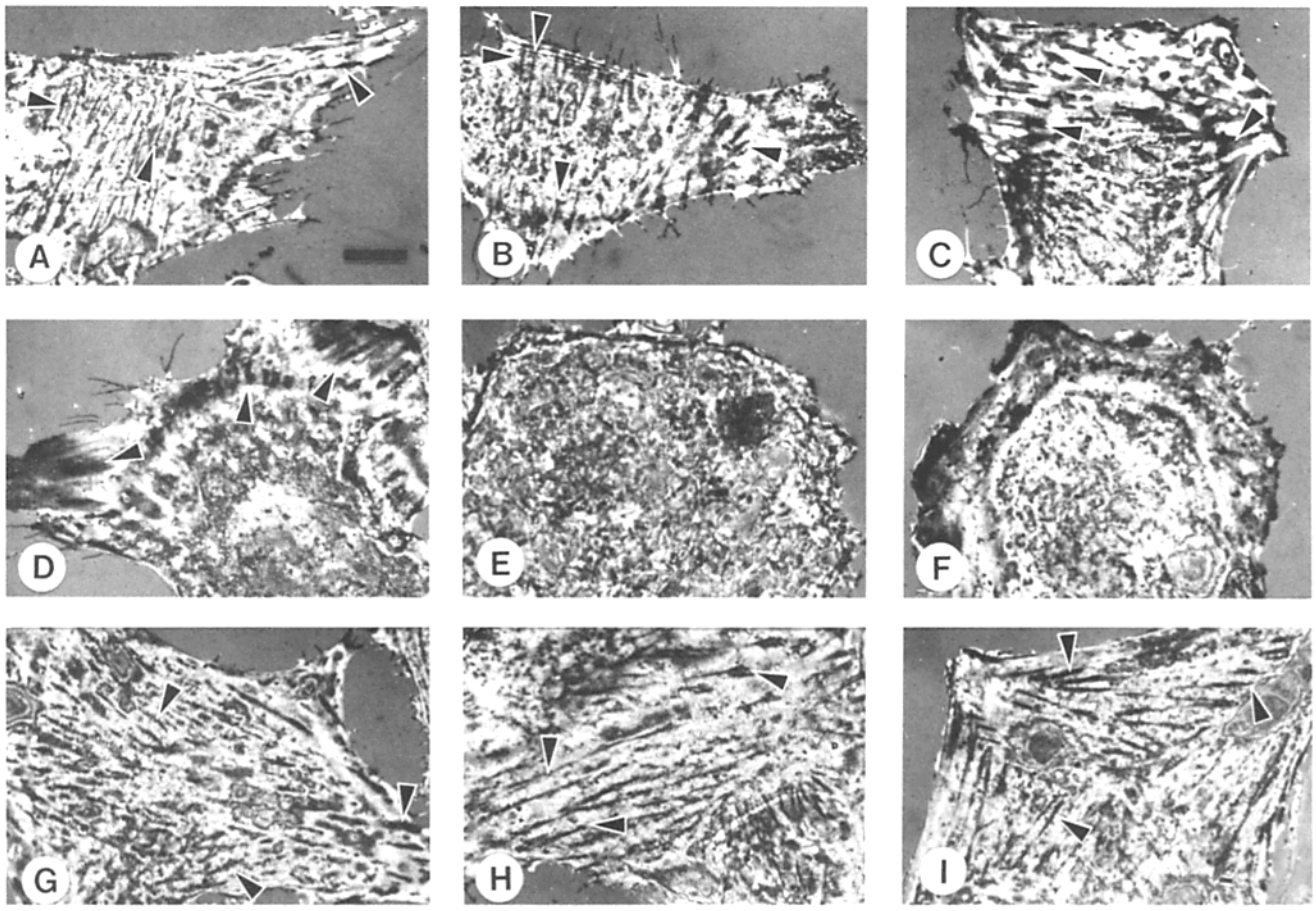

Figure 8. IRM of FC formation in fibroblasts cultured for 2 h on substrata coated with intact FN, or its components. NRK rat fibroblasts (*A–C*), Balb/c 3T3 mouse fibroblasts (*D–F*), or Nil 8 hamster fibroblasts (*G–I*), were seeded onto coverglasses preincubated with native FN (5 μg/ml) (*A*, *D*, and *G*), 75-kD FN cell-binding domain (5 μg/ml) (*B*, *E*, and *H*), or derivatized with Gly-Arg-Gly-Asp-Ser-containing peptide 1 (*C*, *F*, and *I*). The cells for this experiment were dissociated using EGTA instead of trypsin and fixed as described in Materials and Methods. All cell lines formed tight FCs (*arrowheads*) on FN, but the Balb/c 3T3 fibroblasts showed no FCs on 75-kD FN cell-binding domain, nor on GRGDS-peptide 1 (*E* and *F*). Bar, 10 μm.

rat kidney and hamster Nil 8 cells) form FCs soon (1–2 h) after seeding on substrates derivatized with either a small synthetic FN peptide containing the cell recognition sequence Arg-Gly-Asp-Ser, or with an f75kD portion of the FN cell-binding domain. These focal adhesion sites, that were seen in serum-free media, appeared in 70–95% of cells at early times in the absence of detectable FN fiber formation at the substrate-binding surface. In addition, the FCs that formed on FN peptide or f75kD were indistinguishable from those that developed on coverslips coated with FN. We (herein) and others (1, 29–31) have shown that peptides containing the Arg-Gly-Asp-Ser (RGDS) FN cell-binding sequence inhibit fibroblast attachment to surfaces coated with FN, or to substrata derivatized with similar RGDS-containing peptides. Also, RGDS peptides competitively inhibit specific cell-surface binding of the 75-kD FN cell-binding domain, with a correlative reduction in cell spreading (1). A monoclonal antibody recognizing the 140-kD FN-binding complex has very similar properties (2, 5). A control peptide, with a Glu for Asp substitution that interferes with binding to the FN receptor (31), did not promote cellular attachment, failed to prevent cellular adhesion on RGDS surfaces, and was completely ineffective in FC induction. Collectively, these data strongly suggest that our substrate-linked RGDS-containing peptide fosters FC formation at early culture times by attaching to cell-surface adhesion receptors. The localization of the 140-kD FN-binding complex around (7), within (10), and adjacent to FCs along microfilament bundles coincident with fibronexuses and matrix contacts (5, 6, 38, 43) supports the hypothesis that the FN receptor probably belongs to this class of receptors. However, recent studies using liposomes indicate that the vitronectin receptor interacts with RGDS-containing peptides linked to substrates, while the FN receptor does not (32). These results suggest that the vitronectin receptor may be the dominant cell-surface adhesion receptor mediating cell attachment. However, the definitive roles played by the FN and vitronectin receptors in FC formation must be determined by further IFM experiments with intact cells. In this regard it is important to note that the kinetics of FC formation, and FC morphology were always indistinguishable on substrata coated with native FN, f75kD cell-binding domain, or RGDS peptide 1. This suggests that whichever class of cell-surface adhesion receptors participates in FC assembly, the outcome is similar, provided that sufficient quantities of receptors are available. Furthermore, our results also suggest that regardless of the relative functions of the FN and vitronectin receptors in the adhesive process, the Arg-Gly-Asp-Ser sequence

of the FN cell-binding domain provides sufficient attachment function for FC development, without a requirement for further cell-binding activity from adjacent FN domains (e.g., heparin-binding regions that have avidity for cell surface heparan sulfate proteoglycans [23]) in certain cell types.

We believe that the formation of FCs on peptide 1–derivatized coverslips is dependent upon the specific recognition of substrate-bound RGDS sequences by cell-surface adhesion receptors for several reasons. First, peptide 2 (which contains a conservative substitution of Glu for Asp) fails to promote FCs in the few cells that adhere to substrata derivatized with it. Second, control coverslips coated with BSA and cross-linked with SPDP alone failed to mediate FC formation in the stray attached cells. Third, soluble RGDS-containing peptide inhibited cellular adhesion to substrata containing the identical peptide cross-linked to its surface. We were also acutely aware of the possibility that the input cells could be degrading the cross-linked BSA substrate, or, that it could spontaneously break down, thus uncovering additional substrate sites for binding to cell-surface ligands. Accordingly, we examined the possibility of substrate metabolism during the time course of cell adhesion, and found that negligible quantities of BSA were released into the medium. Furthermore, all adhesion experiments were performed in media containing 1 mg/ml BSA in order to block any newly uncovered substrate adhesion sites. We therefore believe that the possibility of substrate metabolism is of little concern in our system.

Interestingly, we observed that the generation of microfilament bundles coincident with FCs at 2 h was not coordinated with FN fiber formation at the substrate-binding surface. At this time, 60–70% of the cells plated on RGDS peptide totally lacked FN fibers, and the FN-positive cells for the most part had FN fibers that were not coincident with microfilament bundles. However, by 4 h in culture on RGDS peptide, the majority of NRK and Nil 8 cells exhibited substrate-attached FN fibers coaligned with complexes of FCs and microfilament bundles. These substrate adhesion complexes undoubtedly are fibronexuses (37, 38, 43) or extracellular matrix contacts (5, 6). The delay in appearance of FN fibers coaligned with the cytoskeleton suggests that in our culture system, sufficiently high concentrations of substrate-linked Arg-Gly-Asp-Ser sequences can substitute for FN function in the early stages of cell attachment and spreading. Several groups have recently observed that the 140-kD FN receptor complex is diffusely distributed in the membranes of mobile cells, but becomes concentrated at FCs and microfilament bundles during adhesion to planar substrates (8, 10). The co-distribution of FN receptors with actin microfilaments and FCs (5, 10) suggests that the 140-kD FN receptor complex might have additional affinities for cytoskeletal proteins. If this is true, surface adhesion receptors of fibroblasts spreading on RGDS-derivatized substrata could undergo patching due to localized binding of the adhesion surface to the high concentrations of RGDS. Such putative clustering of receptors might then provide transmembrane nucleation centers for development of FCs and microfilament bundles. Once formed at 1–2 h, attachment of these contact sites to the substratum might be stabilized by the deposition of FN fibers at the adhesive surface by 4 h. According to this model, formation of fibronexuses or extracellular matrix contacts would strengthen but not initiate cellular adhesion to RGDS-derivatized surfaces.

The finding that fibroblasts cultured 4 h on coverslips derivatized with RGDS peptide formed prominent substrate-attached FN-containing fibers, whereas cells seeded on coverslips coated with intact FN did not, raises the important question of how substrate composition affects the synthesis and deposition of extracellular FN. Recently, preconfluent cultures of arterial smooth muscle cells on FN-coated substrates were shown to synthesize 50% less fibronectin compared with similar cultures on albumin-coated surfaces (17). This observation suggests that the cells are capable of recognizing specific extracellular matrix molecules and appropriately regulating their synthesis via a negative feedback mechanism. If this suggestion is correct, our cells cultured on FN surfaces probably synthesized insufficient FN to form extracellular FN fibers before 8 h. Alternatively, cultures on RGDS peptide 1 synthesize FN fibers despite the apparent binding of Arg-Gly-Asp-Ser sequences to their receptors. This result implies that signals from FN domains in addition to the cell-binding region are required to inhibit FN synthesis.

Our initial data from NRK and Nil 8 fibroblasts were at variance with recent studies in which human or Balb/c 3T3 mouse fibroblasts developed FCs on substrates coated with intact FN, but failed to form FCs on glass covered with purified cell-binding domain of FN (19, 21, 46). Therefore, we also assessed the capacity of Balb/c 3T3 mouse fibroblasts to form FCs on coverslips coated with either FN, the 75kD FN cell-binding domain, or with RGDS-containing peptide 1. This mouse cell line was unable to form significant numbers of FCs on f75kD or RGDS peptide 1 substrata, while both the NRK and Nil 8 fibroblasts showed substantial numbers of FCs on these surfaces. In addition, only 40% of the Balb/c 3T3 cells developed FCs on coverslips coated with native FN, whereas 95–100% of the NRK and Nil 8 fibroblasts plated on this substratum contained FCs 2 h after seeding. These results suggest that the Balb/c 3T3 fibroblasts are deficient in their ability to adhere to FN substrates relative to the rat and hamster cell lines, perhaps because they have fewer functional FN receptors. Furthermore, we hypothesize that the substrates derivatized with high concentrations of Arg-Gly-Asp-Ser cell-surface recognition sequence bind sufficient quantities of adhesion receptors such that the apparent need of certain cells for the heparin-binding domain in FC formation (46) is circumvented. Therefore, the requirement for additional portions of the FN molecule outside the cell-binding domain in the process of substrate attachment may be due to cell-type differences in the functioning of surface adhesion receptors.

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