Immobilized Amines and Basic Amino Acids as Mimetic Heparin-binding Domains for Cell Surface Proteoglycan-mediated Adhesion*

Stephen P. Massia§ and Jeffrey A. Hubbell¶

From the §Department of Chemical Engineering and ¶Division of Biological Sciences, University of Texas, Austin, Texas 78712-1062

*This work was supported by Grants BCS-9057641 and ECS-8915178 from the National Science Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed.

Cells attach to the extracellular matrix in vivo and to culture substrates in vitro by interaction with cell adhesion proteins such as fibronectin, laminin, and vitronectin. These proteins have specific binding domains for extracellular matrix structural components such as collagen, elastin, and proteoglycans. Other domains in cell adhesion proteins mediate the interaction of these proteins with cells to promote cell attachment. An important sequence in the cell-binding domains of cell adhesion proteins is the tripeptide Arg-Gly-Asp (RGD) (1). This tripeptide sequence binds to specific cell surface receptors collectively known as the integrin superfamily (1–8). Integrins are transmembrane heterodimeric glycoproteins which bind to RGD signals extracellularly and coordinate the formation of focal contacts and intracellular stress fibers in attached and spreading cells (9).

Another class of cell adhesive domains found in cell adhesion proteins is known as the heparin-binding domain. These domains have affinities for heparin and other classes of sulfated glycosaminoglycans (GAGs)† including chondroitin 4-sulfate, dermatan sulfate, and heparan sulfate (10). Cell membrane-associated proteoglycans carry extracellular heparan sulfate and/or chondroitin sulfate chains and can be involved in cell adhesion via interactions with heparin-binding domains of cell adhesion molecules (11). These cell surface proteoglycans functioning as adhesion receptors facilitate the organization of actin into stress fibers and the formation of focal contacts in spreading cells (12–14).

Recent studies of protein-GAG interactions have revealed that binding occurs between specific domains of clustered positively charged basic amino acid residues of the protein and negatively charged sulfated and carboxylate groups of the GAG (10). Cardin and Weintraub (15) suggested that a GAG-binding domain in the carboxyl terminus of fibronectin was contained in the sequence PRRARV. Keely Haugen et al. (16) and McCarthy et al. (17) used synthetic peptides to demonstrate that the sequence YEKPGSPREVVPRPGV, derived from the carboxyl-terminal heparin-binding domain of fibronectin, bound heparin, and promoted cell adhesion. Cardin and Weintraub (15) proposed that the sequence RPSSLAKKQFRHRNKVRGYSQREGSVGR, based on a heparin-binding fragment described by Suzuki et al. (18) near the carboxyl terminus of vitronectin, forms a stable complex with heparin oligosaccharides. Three heparin-binding sites have been described for laminin. Kouzi-Koliakos et al. (19) demonstrated that the sequence RIQLNLKTLNRKFKV had heparin-binding activity. Charonis et al. (20) described the laminin-based sequence RYVVLPRVCFKGMNNYTVR which bound heparin and promoted cell adhesion.

Carbon and Weintraub (15) identified a consensus sequence, XBBXBX (X = hydrophobic, B = basic), that is present in many known heparin-binding proteins. Bober Barkalow and Schwarzauer (21) demonstrated that site-directed mutagen-

† The abbreviations used are: GAG, glycosaminoglycan; HFF, human foreskin fibroblast; ESCA, electron spectroscopy for chemical analysis; EDA, ethylenediamine; NMAEA, N-methylaminoethylamine; EA, ethylamine; NNDMAEA, N,N-dimethylaminoethylamine; NNDMAET, N,N-dimethylaminoethanethiol; PFBA, pentafluorobenzaldehyde; PBS, phosphate-buffered saline; EGTA, (ethylenediamine(oxyethylenenitrilo))tetraacetic acid; BSA, bovine serum albumin; CHX, cycloheximide.

10133
esis of adjacent arginines (charged residues were substituted with uncharged residues) in such a consensus sequence found within a heparin-binding domain of fibronectin (PRRGRV) reduced heparin binding by 98%. Therefore, cell surface proteoglycan-mediated adhesion could occur by ionic interactions between the cell surface proteoglycans and the positively charged basic residues found within heparin-binding domains of cell adhesion proteins.

It has been observed that improved cell attachment and growth of fibroblasts and dissociated neurons could be obtained by coating culture substrates with polymers of basic amino acids such as polylysine and polyornithine (22–24). More recently it has been shown that coating substrates with synthetic polymeric amines such as polyethyleneimine (26) and substrate surfaces covalently bound with diamines and triamines (25) can support in vitro neuronal attachment and growth as well as polylysine and polyornithine coatings. Nitrogenated tissue culture plastic with a net positive charge (Primaria; Falcon) has also been developed as a cell culture substrate which promotes improved cell adhesion. It was generally thought that these substrate surface treatments form a molecular layer of positively charged residues which interact with negatively charged lipids or polysaccharides on the cell surface (25). It has recently been shown that cell attachment to Primaria, in the absence of serum proteins, could be inhibited up to 50% by adding heparin to the culture medium (27). This evidence suggested that cell surface proteoglycans interact with positively charged groups on the surfaces of Primaria to promote cell adhesion to these substrates and more generally that positively charged substrate surfaces promote cell adhesion and spreading mediated by cell surface proteoglycans.

In this study, we investigated the ability of covalently immobilized amines and basic amino acids to promote human foreskin fibroblast (HFF) attachment, spreading, and cytoskeletal organization in the absence of serum. Specific orientation of the surface immobilized amines and diamines was demonstrated by electron spectroscopy for chemical analysis (ESCA). Specific orientation of the basic amino acids was obtained by using amine protective chemistry. The interaction of cell surface proteoglycans with these substrates was demonstrated by inhibiting cell attachment with GAG-degrading enzymes. We sought to define particular amines which would promote cell surface proteoglycan-mediated cell adhesion and as such serve as mimetic structures of the heparin-binding domains of cell adhesion proteins.

**EXPERIMENTAL PROCEDURES**

**Amines, Diamines, and Amino Acids—**Ethylendiamine (EDA), N-methylaminoethylamine, (NMAEA), ethylamine (EA), and N,N-dimethylaminoethylamine (NNDMAEA) were purchased from Aldrich. N,N-dimethylaminethanol (NNDMAET) was obtained from Pfaltz and Bauer. Arginine and N-ε-t-boc-lysine were purchased from Sigma.

**Preparation of Amine- and Diamine-grafted Substrates—**A three-step chemical process was used for substrate preparation (28). In the first step, glass was rendered relatively poorly cell-adhesive by modification with a silylating agent to produce what is called glyophase glass. In the second step, the glyophase glass was activated with a sulfonyl chloride, and in the final step, the activated glyophase glass was coupled to the amines, diamines, or amino acids via primary amine or thiol moieties on these molecules (see Fig. 1 for the specific amines and diamines).

Specifically, glass coverslips (18 by 18 mm; Thomas) were soaked in 0.5 M NaOH for 2 h, rinsed in deionized water, and immersed in an aqueous solution (1%, pH 5.5) of (3-glycidoxypropyl)trimethoxysilane (Fisher Scientific Inc.). The preparation was heated and maintained at 90 °C for 2 h. The coverslips were then rinsed in deionized water to remove unreacted silane. 1 mM HCl was added to the dish containing the silylated glass coverslips, and this preparation was heated at 90 °C for 1 h to convert the oxirane moieties on the derivatized glass to glycol groups (glyophase glass). Superficially dry glyophase glass coverslips were rinsed with dry dioxane (dried over molecular sieve 4Å; Fisher). To 1 ml of dry dioxane, 200 μl of dry 0.5 M NaOH and 100 μl of dry 0.5 M NaOH were added. A minimal volume of 1 M NaOH was added to the upper surface of each glyophase glass coverslip placed in a glass Petri dish. The reaction was allowed to proceed for 10 min at room temperature, after which the coverslips were rinsed in 1 mM hydrochloric acid and finally rinsed in 0.2 M sodium bicarbonate buffer at pH 8.5 (coupling buffer). 1 mM sodium sulfite was added to the coupling buffer containing 0.2 M β-mercaptoethanol for 2 h, which effects removal of the unreacted teryl groups with a nonadsorptive moiety.

**Substrate Surface Analysis and Characterization—**Predictions of the orientation of the covalently immobilized mono- and diamines (see Fig. 1 for each immobilized amine and its expected orientation) were made based on the relative reactivities of primary (1°), secondary (2°), tertiary (3°) amines and thiol groups (SH) with the surface-immobilized sulfonfyl esters. The relative reactivities ordered from highest to lowest are as follows: SH > 1° > 2° > 3° (29). Sulfonfyl esters are formed by the reaction of the sulfonfyl chloride (teryl chloride) and glyophase glass. Amines and thiol groups displace the surface-bound teryl esters and form a covalent bond with the silylated glass surface. Diamines containing one 1° amine and one 2° or 3° amine separated by two carbon atoms would be expected to bind to the surface via the 1° amine, leaving a free 2° or 3° amine with a two-carbon spacer arm.

It was necessary to verify some of the expected orientations of the immobilized multifunctional species. Specifically, surface analysis was performed to demonstrate that EDA bound to the activated substrates only by one end, thus leaving one 1° amine free; and that the NMAEA bound by the 1° amine, thus leaving the 2° free. Amino- and thiolimmobilized glass substrates were made based on the relative reactivities of primary (1°), secondary (2°), tertiary (3°) amines and thiol groups (SH) with the surface-immobilized sulfonfyl esters. The relative reactivities ordered from highest to lowest are as follows: SH > 1° > 2° > 3° (29). Sulfonfyl esters are formed by the reaction of the sulfonfyl chloride (teryl chloride) and glyophase glass. Amines and thiol groups displace the surface-bound teryl esters and form a covalent bond with the silylated glass surface. Diamines containing one 1° amine and one 2° or 3° amine separated by two carbon atoms would be expected to bind to the surface via the 1° amine, leaving a free 2° or 3° amine with a two-carbon spacer arm.

It was necessary to verify some of the expected orientations of the immobilized multifunctional species. Specifically, surface analysis was performed to demonstrate that EDA bound to the activated substrates only by one end, thus leaving one 1° amine free; and that the NMAEA bound by the 1° amine, thus leaving the 2° free. Amino- and thiolimmobilized glass substrates were made based on the relative reactivities of primary (1°), secondary (2°), tertiary (3°) amines and thiol groups (SH) with the surface-immobilized sulfonfyl esters. The relative reactivities ordered from highest to lowest are as follows: SH > 1° > 2° > 3° (29). Sulfonfyl esters are formed by the reaction of the sulfonfyl chloride (teryl chloride) and glyophase glass. Amines and thiol groups displace the surface-bound teryl esters and form a covalent bond with the silylated glass surface. Diamines containing one 1° amine and one 2° or 3° amine separated by two carbon atoms would be expected to bind to the surface via the 1° amine, leaving a free 2° or 3° amine with a two-carbon spacer arm.

It was necessary to verify some of the expected orientations of the immobilized multifunctional species. Specifically, surface analysis was performed to demonstrate that EDA bound to the activated substrates only by one end, thus leaving one 1° amine free; and that the NMAEA bound by the 1° amine, thus leaving the 2° free. Amino- and thiolimmobilized glass substrates were made based on the relative reactivities of primary (1°), secondary (2°), tertiary (3°) amines and thiol groups (SH) with the surface-immobilized sulfonfyl esters. The relative reactivities ordered from highest to lowest are as follows: SH > 1° > 2° > 3° (29). Sulfonfyl esters are formed by the reaction of the sulfonfyl chloride (teryl chloride) and glyophase glass. Amines and thiol groups displace the surface-bound teryl esters and form a covalent bond with the silylated glass surface. Diamines containing one 1° amine and one 2° or 3° amine separated by two carbon atoms would be expected to bind to the surface via the 1° amine, leaving a free 2° or 3° amine with a two-carbon spacer arm.

It was necessary to verify some of the expected orientations of the immobilized multifunctional species. Specifically, surface analysis was performed to demonstrate that EDA bound to the activated substrates only by one end, thus leaving one 1° amine free; and that the NMAEA bound by the 1° amine, thus leaving the 2° free. Amino- and thiolimmobilized glass substrates were made based on the relative reactivities of primary (1°), secondary (2°), tertiary (3°) amines and thiol groups (SH) with the surface-immobilized sulfonfyl esters. The relative reactivities ordered from highest to lowest are as follows: SH > 1° > 2° > 3° (29). Sulfonfyl esters are formed by the reaction of the sulfonfyl chloride (teryl chloride) and glyophase glass. Amines and thiol groups displace the surface-bound teryl esters and form a covalent bond with the silylated glass surface. Diamines containing one 1° amine and one 2° or 3° amine separated by two carbon atoms would be expected to bind to the surface via the 1° amine, leaving a free 2° or 3° amine with a two-carbon spacer arm.

It was necessary to verify some of the expected orientations of the immobilized multifunctional species. Specifically, surface analysis was performed to demonstrate that EDA bound to the activated substrates only by one end, thus leaving one 1° amine free; and that the NMAEA bound by the 1° amine, thus leaving the 2° free. Amino- and thiolimmobilized glass substrates were made based on the relative reactivities of primary (1°), secondary (2°), tertiary (3°) amines and thiol groups (SH) with the surface-immobilized sulfonfyl esters. The relative reactivities ordered from highest to lowest are as follows: SH > 1° > 2° > 3° (29). Sulfonfyl esters are formed by the reaction of the sulfonfyl chloride (teryl chloride) and glyophase glass. Amines and thiol groups displace the surface-bound teryl esters and form a covalent bond with the silylated glass surface. Diamines containing one 1° amine and one 2° or 3° amine separated by two carbon atoms would be expected to bind to the surface via the 1° amine, leaving a free 2° or 3° amine with a two-carbon spacer arm.
tained in Dulbecco’s modified Eagle’s medium supplemented with
10% fetal calf serum, 400 units/ml penicillin, and 400 µg/ml strepto-
tomyycin (all from GIBCO Bethesda Research Laboratories) and were
incubated at 37 °C in a humidified 5% CO₂ atmosphere.

Cell Spreading Assays—Cells harvested for experiments were
rinsed twice with Ca²⁺- and Mg²⁺-free PBS and then incubated in
0.5% BSA in 1:1 PBS-Tyrodes (pH 7.4) for 1 h at 37 °C to prevent
further spreading. Cells were collected by centrifugation and resuspended in serum-free me-
dium, which consisted of Dulbecco’s modified Eagle’s medium with 4
mg/ml of heat-inactivated (90 °C, 10 min) bovine serum albumin
(BSA; Sigma) and antibiotics. Heat treatment of the albumin achieves
the denaturing of contaminating cell adhesion proteins without ad-
versely affecting the albumin (13). The substrates were seeded at a
density of 10,000 cells/cm², and cells were allowed to attach and
spread at 37 °C in 5% CO₂. An inverted microscope (Fluovert, Leitz)
equipped with phase contrast objectives and a high resolution video
camera (67M series, Dage-MTI) was used to visualize spreading cells
after 4-h incubation. Random fields of adherent cells were chosen for
video recording and at least 100 cells were analyzed on each substrate.

Adherent cells were observed and categorized into four morphological
types, as described by Massia and Hubbell (31): Type I, spheroid with
no filopodia; Type II, spheroid with one to two filopodia; Type III,
spheroid with two filopodia; Type IV, morphologically similar to fibro-
blasts but flattened and polygonal or flattened with pseudopodia. The population of adherent
cells analyzed on each substrate was scored according to the above
criteria, and the percentage of each morphological type in the sample
population was determined for each substrate 4 h after seeding. The percentage of attached cells which were spread (Type I or II)
was obtained by multiplying the ratio of spread cells to the total number
of attached cells (Types I–IV) by 100; nonadherent cells were discrim-
inated from adherent ones by gently shaking the dish.

The effects of secreted extracellular matrix proteins on HFF
spreading on these amine-grafted substrates were examined. Cells
were preincubated in the presence or absence of the protein synthesis
inhibitor cycloheximide (CHX; 20 µg/ml) for 30 min prior to inoculation
on the substrates. The percentage of the attached cells which were
spread was determined 4 h after inoculation as described above.

Cell viability was determined 4 h after seeding cells on the various
substrates and tissue culture plastic. A two-color fluorescence cell
viability assay (Live/Dead Viability/Cytotoxicity Assay, Molecular
Probes) was employed to determine the relative cytotoxicities of the
substrates. Live cells stain positively with fluorescein by uptake of
nonfluorescent fluorescein acetate and conversion to fluorescent flu-
orescein. Dead cells stain positively by uptake of propidium bromide.
Tissue culture plastic served as a nontoxic reference material. Cells
were stained according to the manufacturer’s instructions and were
visualized on a Fluovert microscope (Leitz) equipped with a 200 ×
Fluor objective. Live and dead cells were counted in each of 10
fields for each substrate. Live cells were visualized using a fluorescein
filter set, and dead cells were visualized from a Texas red and a
rhodamine filter set. The percentage of viable cells for each field was determined by
multiplying the ratio of live cells to total cells (live cells plus dead
cells) per field by 100. The mean percentage was determined from 10
fields sampled for each substrate.

In cell spreading assays in which heparinase or chondroitinase
enzymes (Sigma) were used, cell suspensions in serum-free medium
were pretreated with the enzymes for 5 min at 37 °C prior to seeding
on the substrates as described in (32). Controls were untreated or
pretreated with heat-inactivated (10 min, 100 °C) enzymes for 5
min at 37 °C prior to seeding. The percentage of spread cells was
determined after 4-h incubation. The presence of nonspecific proteases
in the chondroitinase ABC preparation was determined by pretreatment
of cells with the enzyme preparation prior to seeding of cells on
substrates containing covalently grafted RGD peptides (28). Cells
attach to these substrates via integrins which are sensitive to some
proteases but are not affected by chondroitinase ABC.

Scanning Electron Microscopy—Samples of adherent cells on
the various substrates were prepared by fixation with 2% glutaraldehyde
in PBS for 1 h. The samples were then rinsed in water and dehydrated in
a graded ethanol series for 10-min exchanges in each of 50, 70, 90,
and 100% alcohol. The samples were dried by sublimation in a high
vacuum desiccator and then sputter-coated with carbon and gold. These
preparations were photographed from a high resolution video camera (Model PVM 1271Q, Sony).

Focal Contact Visualization—Immunofluorescence microscopy was
performed to observe the distribution of vinculin (a protein which
localizes within focal contacts) in spread cells on fibronectin-coated
and NNDMAEA-grafted substrates. The preparations were counter-
stained with rhodamine-conjugated phalloidin to visualize microfila-
ment bundle formation. Focal contacts in spread cells were visualized
by interference reflection microscopy. The primary antibody used in
these studies, monoclonal mouse anti-human vinculin, was obtained
from Chemicon. The secondary antibody used was goat anti-mouse
type-III, rhodamine-conjugated sheep anti-mouse IgG (from Accurate
Chemical and Scientific Corporation). At various incubation periods, cells on
derivatized glass coverslips were rinsed with PBS and fixed with 4%
formaldehyde (prepared fresh from paraformaldehyde) in PBS for 10
min. Trapped cells were permeabilized with 0.1% Triton X-100 in PBS
for 2 min. The samples were then briefly rinsed in PBS and washed
in 2 mg/ml BSA in PBS (BSA-PBS) for 5 min. Samples were
incubated with a mixture of primary antibody (diluted 1:100 in BSA-
PBS) and 900 ng/ml rhodamine-conjugated phalloidin for 45 min at
37 °C. After the first incubation, samples were washed twice in BSA-
PBS for 5 min each wash. The samples were then incubated with
secondary antibody (diluted 1:50 in BSA-PBS) for 45 min at 37 °C.
After two 5-min washes in BSA-PBS, the samples were mounted on
glass slides with 1:1 glycerol/PBS and sealed with nail polish. Cells
adherent to fibronectin-coated glass substrates were stained using
this protocol and served as a reference control for typical spread cell
features such as localization of vinculin within focal contacts
and termination of stress fibers in these regions of contact. Fibronectin-
coated glass was prepared by incubating untreated glass coverslips
with 20 µg/ml plasma fibronectin (Sigma) in PBS for 1 h and
subsequently with 1 mg/ml BSA in PBS for 30 min. These substrates
were rinsed in PBS prior to seeding with cells. The preparations were
viewed on a Fluovert microscope equipped with a 100 × Pl Fluor
objective and a 100-watt mercury lamp. Actin microfilaments, vin-
culin, and focal contacts were visualized in the same field using the
appropriate filter set for each technique. These preparations were
photographed from a high resolution video monitor after digital
contrast enhancement of video images.

RESULTS

Surface Analysis of Amine- and Diamine-grafted Sub-
strates—Derivatization of substrates with tretyl chloride, which
reacts with 1° and 2° amines, resulted in high F/Si peak area ratios
(0.22 and 0.18) for EDA- and NMAEA-grafted substrates. These values indicated that these
substrates were in highly reactive 1° and 2° amine moieties and were
derivatized with similar amounts of the amines. Grafted EA, which contains a single surface-bound 2° amine
per molecule, had a low F/Si peak area ratio (0.035), indicating that the surface-bound (close to the glass, not pendant) 2° amine was not very accessible to the tretyl chloride. Surface-
coupled NNDMAEA and NNDMABT, which were predicted to contain only free unreactive 1° amines, also demonstrated low F/Si peak area ratios (0.027 and 0.029, respectively). The amine on
grafted N-ethylmethyamine, the blocking agent used to dis-
place unreacted tretyl groups, was predicted not to react with
tretyl chloride and demonstrated a low F/Si ratio (0.011). The
basal levels of F on the EA-, N-ethylmethyamine-, NNDMAE-, and NNDMAB-grafted surfaces could be
due to the reaction of tretyl chloride with remaining hydroxyl
groups in the base glycophasel glass material.
PFBA derivatizations, where only 1° amines are reactive, were performed on EDA-, NMAEA-, and NNDMAEA-grafted substrates. A large F/Si peak area ratio of 0.54 was observed on EDA-grafted substrates, indicating the presence of 1° amines. PFBA-treated NMAEA- and NNDMAEA-grafted substrates demonstrated low F/Si peak area ratios (0.11 and 0.12, respectively), demonstrating a lack of 1° amines moieties on the surface. The basal level of F/Si was likely due to the reaction of PFBA with remaining hydroxyl groups in the base glycophase glass material.

These results were all consistent with the expected orientations shown in Fig. 1. In particular, EDA coupling was shown to produce pendant 1° amines (tresyl-positive and PFBA-positive; it could have coupled to the substrate surface at both ends, to give tresyl-positive but PFBA-negative); and NMAEA treatment produced pendant 2° amines (tresyl-positive, PFBA-negative; it could have bound by the 2° amine instead, to give tresyl-negative and PFBA-positive). The other species could only react in one orientation, and the surface analytical results verified the expected configuration.

Cell Spreading Behavior and Cytoskeletal Organization on the Various Adhesion Substrates—The covalently coupled amines promoted HFF adhesion (50-60% of the input cells initially in suspension) in the absence of serum. NNDMAEA-derivatized substrates supported the highest degree of cell spreading, with 73 7% of the adherent cells analyzed having type III and IV morphologies (actively spreading cells; Table I). The role of the surface-bound 2° amines in the diamine-grafted substrates was investigated by coupling EA (to add only the surface-bound 2° amine) and NNDMAET (to remove it) to obtain the structures shown in Fig. 1. NNDMAET-grafted substrates, where the substrate surface contained only free 3° amines, supported actively spreading cells in only 3 7% of adherent cell population (Table I). EA-grafted substrates, which contained only the surface-bound 2° amine, promoted active spreading in only 18 4% of the adherent cell population (Table I). Covalently bound arginine and lysine supported cell spreading which was comparable to that on NNDMAEA-grafted substrates (see Fig. 4). Specifically, 74 5% of the adherent cells analyzed displayed types III and IV morphologies on lysine-grafted substrates and 78 6% for arginine-grafted substrates (Table I).

To determine the effect of secreted cellular proteins on HFF spreading on the amine-grafted substrates, cells were incubated on the various substrates in the presence or absence of CHX. Cells were scored by morphological types which represent the extent of cell spreading, and the percentage of each type was determined for each substrate. Type I morphologies are spheroid with no filapodia; Type II are spheroid with one to two filapodia; Type III are spheroid with greater than two filapodia; and Type IV are flattened and polygonal or flattened with pseudopodia. Samples in individual experiments were run in triplicate and averaged, and mean values were determined from averaged values of two separate experiments; for each sample, at least 100 cells were analyzed.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Type I</th>
<th>Type II</th>
<th>Type III</th>
<th>Type IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDA</td>
<td>69 ± 9</td>
<td>12 ± 2</td>
<td>9 ± 2</td>
<td>10 ± 4</td>
</tr>
<tr>
<td>NMAEA</td>
<td>79 ± 1</td>
<td>14 ± 2</td>
<td>4 ± 1</td>
<td>3 ± 4</td>
</tr>
<tr>
<td>NNDMAEA</td>
<td>15 ± 1</td>
<td>12 ± 3</td>
<td>25 ± 2</td>
<td>48 ± 4</td>
</tr>
<tr>
<td>NNDMAET</td>
<td>83 ± 7</td>
<td>14 ± 9</td>
<td>2 ± 1</td>
<td>1 ± 0</td>
</tr>
<tr>
<td>EA</td>
<td>69 ± 4</td>
<td>13 ± 1</td>
<td>13 ± 2</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>Arg</td>
<td>12 ± 2</td>
<td>10 ± 1</td>
<td>36 ± 3</td>
<td>42 ± 3</td>
</tr>
<tr>
<td>Lys</td>
<td>14 ± 2</td>
<td>12 ± 1</td>
<td>30 ± 2</td>
<td>44 ± 3</td>
</tr>
</tbody>
</table>

* ± S.E.

FIG. 1. Structure of the functional groups that were bound to substrate surfaces in this study. The charge on the amine and carboxyl groups refers to the ionization state of the group at pH 7.4.
toxic than tissue culture plastic (70–75% viability on all substrates including tissue culture plastic), thus eliminating the possibility that differences in cell spreading were really secondary to differences in cell viability.

SEM confirmed that type I and II morphologies were prevalent in adherent cells on EDA-, NMAEA-, NNDMAET-, and EA-derivatized substrates (Fig. 3, A–D). NNDMAEA-, arginine-, and lysine-derivatized substrates supported adherent cells with predominantly types III and IV morphologies (Fig. 3, E–G).

Rhodamine-phalloidin staining for F-actin in spread cells demonstrated little or no microfilament bundle organization in spread cells on EDA-, NMAEA-, NNDMAET-, and EA-derivatized substrates after 4-h incubation (Fig. 4, A–D). However short, thick bundles were observed in the periphery of most spread cells on NNDMAEA-grafted substrates (Fig. 4E). Some spread cells on NNDMAEA-grafted substrates formed long, thin microfilament bundles (Fig. 5D). Long, thin bundles were observed in all spread cells on arginine- and lysine-derivatized substrates (Fig. 4, E–G).

Although microfilament bundle networks were observed within spread cells on NNDMAEA-, Arg-, and Lys-grafted substrates, they were not as extensive as the typical stress fiber networks observed within spread cells after 4-h incubation on fibronectin-coated substrates (Fig. 5, A and D). Interference reflection microscopy and antivinculin staining demonstrated focal contact formation within spread cells on fibronectin (Fig. 5, B and C), however these visualization techniques revealed no focal contacts within spread cells on the NNDMAEA-grafted substrates (Fig. 5, E and F).

Effects of Heparinase and Chondroitinase ABC on HFF Spreading—HFFs were inoculated onto NNDMAEA-, lysine-, and arginine-grafted glass substrates in the presence of 2 units/ml of heparinase or 10 units/ml chondroitinase ABC. This level of heparinase was 100 times in excess of levels reported to degrade greater than 85% of cell surface heparin sulfate (32). The level of chondroitinase ABC used in this study was 100 times in excess of levels reported to remove greater than 60% of cell-associated chondroitin sulfate (33). Spreading was inhibited by 80–85% on all three substrates after treatment with chondroitinase ABC, as shown in Table II. Heparinase had no effect on cell spreading on NNDMAEA-grafted substrates and approximately 25% inhibition of cell spreading on lysine- and arginine-grafted substrates. Pretreatment of cells with heat-inactivated enzymes failed to inhibit spreading on all substrates. The chondroitinase ABC preparation did not interfere with integrin-mediated cell spreading by nonspecific proteolysis of these receptor proteins, since it was observed that chondroitinase ABC pretreatment of HFFs did not affect spreading on RGD-grafted substrates (Table II).

DISCUSSION

It has been well established that proteoglycans are involved in cell attachment and spreading. From biochemical analyses of cell-substrate adhesion sites, Culp et al. (34, 35) provided the first evidence that heparin sulfate proteoglycans played a direct role in forming adhesive bonds on fibronectin-coated
Fig. 4. Epifluorescence microscopy of rhodamine-phalloidin-stained preparations of HFFs spread on the various substrates after 4-h incubation. Substrates: A, EDA; B, NMAEA; C, NNDMAE; D, EA; E, NNDMAEA; F, arginine; G, lysine. Little or no cytoskeletal organization was observed in spread cells on substrates which promoted suboptimal spreading (substrates A-D). Short, thick actin microfilament bundles were observed in spread cells on NNDMAEA-derivatized substrates (E). Long, thin bundles were observed in spread cells on arginine- and lysine-grafted substrates (F, G). Scale bar = 20 μm.

Substrates. Other studies demonstrated high affinity proteoglycan binding and specific proteoglycan-binding domains in cell adhesion proteins (reviewed in Ref. 10). Studies by Laterra et al. (32, 36) first demonstrated that cell adhesion and spreading without focal contact formation could be mediated by the heparin-binding protein platelet factor 4. There has been some evidence, for particular cell types, that the cell-binding fragment of fibronectin which contains the RGD sequence is not sufficient for focal contact formation in spread cells (12, 13). The study by Woods et al. (13) demonstrated that focal contacts could form in spread normal rat kidney fibroblasts if fibronectin cell-binding fragments and heparin-binding fragments were both adsorbed to substrates.

In previous studies, we have sought to develop a method to covalently immobilize upon substrate surfaces small peptides based on active sequences of cell-binding domains of cell adhesion proteins to promote cell adhesion and spreading (28, 31). These covalently bound short peptide sequences were more stable to cellular proteolysis and thermal degradation than adsorbed cell adhesion proteins or adsorbed proteins conjugated with adhesion peptides, since protein desorption was eliminated and the active groups were not as exposed to soluble proteases. Although we observed that covalently immobilized GRGDY was sufficient for focal contact and stress fiber formation in spread HFFs (31), we have observed that some other cell types do not form these elements on substrates containing only cell-binding domain peptides. These cell types probably require secondary binding sites from cell adhesion proteins such as heparin-binding domains. Therefore, we sought to develop small synthetic molecules which promote cell surface proteoglycan-mediated cell adhesion. This may be advantageous in some cases over binding peptide sequences which serve as heparin-binding domains, since these are frequently rather long sequences.

In this study we demonstrated that amine-containing organic molecules and basic amino acids covalently grafted to substrate surfaces could promote cell surface proteoglycan-mediated adhesion and spreading. The amine moieties, which are essentially all charged at physiological pH, can interact with negatively charged sulfate and carboxylate groups of sulfated proteoglycans in a manner similar to basic amino acid clusters of heparin-binding domains in cell adhesion proteins. Orientation of the covalently grafted amines and diamines was predicted based on the immobilization chemistry employed (29) and was confirmed by ESCA analysis.

We observed that all of the amines and diamines employed in this study were not equally effective in promoting cell surface proteoglycan-mediated adhesion and spreading. The difference in the extent of spreading on these substrates was not due to differences in relative cytotoxicities of the various substrates, since viabilities of spread cells on these substrates was comparable to that on tissue culture plastic. Furthermore, secreted cellular proteins did not play a significant role in cell spreading on these substrates since CHX treatment of cells had minimal effects on cell spreading (Fig. 2). Cell spreading was most effective on substrates containing covalently grafted NNDMAEA, where the free amine was a
FIG. 5. Rhodamine-conjugated phallolidin staining (A, D), interference reflection microscopy (B, E), and antivinculin staining (C, F) for field-matched visualization of focal contacts and microfilament bundles within spread cells on fibronectin-coated (A-C) and NNDMAEA-grafted (D-F) substrates. An extensive microfilament bundle network (A) and focal contacts (B, C) were observed within spread cells on the fibronectin-coated substrates. On this control substrate, the typical cell spreading response was observed. Stress fibers were observed to terminate at focal contact regions and vinculin was localized within focal contacts (one example of these structures is indicated by arrowheads). Some microfilament bundles were observed within spread cells on NNDMAEA-grafted substrates (D), but no focal contacts were evident by interference reflection microscopy (E) or antivinculin staining (F). Scale bar = 20 μm.

<table>
<thead>
<tr>
<th>Enzyme concentration</th>
<th>Heat-treated enzyme</th>
<th>NNDMAEA-grafted glass</th>
<th>Lysine-grafted glass</th>
<th>Arginine-grafted glass</th>
<th>GRGDY-grafted glass</th>
</tr>
</thead>
<tbody>
<tr>
<td>No enzyme</td>
<td></td>
<td>80 ± 5</td>
<td>84 ± 6</td>
<td>78 ± 3</td>
<td>55 ± 3</td>
</tr>
<tr>
<td>2 units/ml heparinase</td>
<td>+</td>
<td>78 ± 6</td>
<td>82 ± 3</td>
<td>76 ± 4</td>
<td>ND*</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>81 ± 3</td>
<td>62 ± 5</td>
<td>56 ± 7</td>
<td>ND</td>
</tr>
<tr>
<td>10 units/ml chondroitinase ABC</td>
<td>+</td>
<td>83 ± 6</td>
<td>79 ± 5</td>
<td>80 ± 3</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>17 ± 4</td>
<td>13 ± 4</td>
<td>16 ± 8</td>
<td>60 ± 4</td>
</tr>
</tbody>
</table>

* ND, not determined.

N,N-dimethyl-substituted 3′ amine separated by two carbon atoms from a surface-bound 2′ amine. Surfaces, which were coupled with NNDMAET and contained only the free 3′ amine, supported very little cell spreading. EA-grafted substrates, which contained only the surface-bound 2′ amine, also promoted little cell spreading. These results suggest that both the free 3′ amine and the surface-bound 2′ amine were important for promoting cell spreading on these substrates.
Kleinfeld et al. have observed that silicon and quartz surfaces covalently bound with diamines and triamines supported neurite outgrowth of dissociated neurons, whereas monoaamine-bound substrates did not promote neurite outgrowth (26).

Staining for F-actin in cells on each amine- and diamine-grafted substrates revealed that microfilament bundles formed only in spread cells on NNDMAEA-grafted substrates (Fig. 4). Short, thick bundles were found in the periphery of most spread cells on this substrate and appeared similar to those observed by Laterra et al. (32), Lark et al. (37), and Izzard et al. (12) on platelet factor 4- and fibronectin heparin-binding fragment-coated substrates. Interference reflection microscopy did not reveal focal contact formation in spread cells on NNDMAEA-grafted substrates, which is indicative of nonintegrin-mediated cell adhesion (Fig. 5, E and F). Our observation of this pattern of microfilament bundle organization and the lack of focal contact formation in spread HFFs on NNDMAEA-grafted substrates suggested that cell spreading on this substrate may be cell surface proteoglycan-mediated.

Further evidence for proteoglycan-mediated cell adhesion and spreading on NNDMAEA-grafted substrates was provided by studies where cells were treated with glycosaminoglycan lyases. Preincubation of cells with chondroitinase ABC inhibited cell spreading on this substrate by 85%, whereas treatment with inhibitory concentrations of heparinase did not affect cell spreading (Table II). The chondroitinase ABC preparation did not interfere with integrin-mediated cell attachment on substrates containing covalently grafted RGD, therefore it was concluded that this enzyme preparation did not nonspecifically lyse all cell surface components which promote cell adhesion (Table II). These results provided direct evidence that NNDMAEA-derivatized substrates promote cell surface proteoglycan-mediated cell spreading specifically via chondroitin sulfate proteoglycans. Studies by Piepkorn and Chapman (27) demonstrated that the presence of heparin or heparin sulfate in the medium inhibited 3T3 and fibrobr-some attachment to Primaria culture plastic substrates; they observed that chondroitin sulfates also inhibited the attachment of myeloma cells to this substrate. The observations of Piepkorn and Chapman (27) provided evidence for cell surface proteoglycan-mediated spreading on a positively charged substrate, however, the surface chemical composition of the Primaria substrates was not well defined. Therefore no conclusions could be made about what substrate surface-bound functionalities were involved in promoting cell spreading.

Since lysine and arginine residues are prevalent in glycosaminoglycan-binding proteins, we investigated the interaction of HFFs with substrates containing α-amine-bound lysine or arginine. We observed that cell spreading and cell viability on lysine- and arginine-grafted substrates were comparable to that on NNDMAEA-grafted substrates (Table I). Secreted cellular proteins did not play a significant role in cell spreading on these substrates, since CHX treatment minimally affected cell spreading on these substrates (Fig. 2). Microfilament bundles in spread cells on lysine- and arginine-grafted substrates were generally longer and thinner than those observed in spread cells on the NNDMAEA-grafted substrates (Fig. 4), however focal contacts were not observed in spread cells on these substrates (data not shown). Inhibition of spreading on lysine- and arginine-grafted substrates by treatment with chondroitinase ABC was comparable to that observed on the NNDMAEA-grafted substrates and confirmed that cell surface proteoglycans were involved in cell spreading to these substrates. A 25% inhibition of cell spreading was observed on the lysine- and arginine-grafted substrates when cells were treated with heparinase (Table II). This result suggested that cell surface heparin sulfate proteoglycans helped mediate cell spreading upon these substrates in addition to cell surface chondroitin sulfate proteoglycans. These studies confirmed the role of basic amino acid residues in mediating interactions of sulfated GAGs with GAG-binding proteins as demonstrated recently by Bober Barkalow and Schwarzauer (21) from sequences within a fibronectin heparin-binding domain.

This study demonstrated and confirmed that positively charged molecules can interact directly with cell surface proteoglycans to mediate cell attachment and spreading. We have demonstrated that the basic amino acid residues lysine and arginine can mediate cell spreading with equal efficacy. Using well defined surface modifications and clearly establishing the orientation of the molecular species on the substrate surfaces, we demonstrated that a particular chemical or physical structure bearing positive charges is necessary for optimal interaction with cell surface proteoglycans. This is to say that it is not merely the charge or the charge density that determines the interaction, but rather the particular molecular configuration of the charged species. It is not clear, however, what fundamental properties of these surface-associated structures govern their biological activity. Such issues require further investigation to be clarified.

Acknowledgments—Special thanks to G. Arrindell, C. LaGraize, T. Du Laney, and M. Guril for technical assistance. We gratefully acknowledge ESCA analyses and interpretations of data provided by M. Arendt.

REFERENCES

Mimetic Heparin-binding Domains