Detachment of Cells from Culture Substrate by Soluble Fibronectin Peptides

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ABSTRACT The synthetic cell attachment-promoting peptides from fibronectin (Pierschbacher, M. D., and E. Ruoslahti, 1984, Nature (Lond.), 309:30-33) were found to detach cultured cells from the substratum when added to the culture in a soluble form. Peptides ranging in length from tetrapeptide to heptapeptide and containing the active L-arginyl-glycyl-L-aspartic acid (Arg-Gly-Asp) sequence had the detaching activity, whereas a series of different peptides with chemically similar structures had no detectable effect on any of the test cells. The Arg-Gly-Asp-containing peptides caused detachment of various cell lines of different species and histogenetic origin. Studies with defined substrates showed that the active peptides could inhibit the attachment of cells to vitronectin in addition to fibronectin, indicating that vitronectin is recognized by cells through a similar mechanism as fibronectin. The peptides did not inhibit the attachment of cells to collagen. However, cells cultured on collagen-coated plastic for 24–36 h, as well as cells with demonstrable type I or type VI collagen in their matrix, were susceptible to the detaching effect of the peptides. These results indicate that the recognition mechanism(s) by which cells bind to fibronectin and vitronectin plays a major role in the substratum attachment of cells and that collagens may not be directly involved in cell-substratum adhesion. Since vitronectin is abundant in serum, it is probably an important component in mediating the attachment of cultured cells. The independence of the effects of the peptide on the presence of serum and the susceptibility of many different cell types to detachment by the peptide show that the peptides perturb an attachment mechanism that is intrinsic to the cells and fundamentally significant to their adhesion.

Studies leading to an understanding of the mechanisms involved in the adhesion of cultured cells to their extracellular matrices will help elucidate such biological phenomena as cell division, embryonic cell migration and sorting, and tumor invasion and metastasis. Fibronectin is a well characterized extracellular structural glycoprotein that interacts with other extracellular matrix molecules and promotes cell attachment and spreading (8, 16, 27). The ability of cells to bind to fibronectin can now be accounted for by the tripeptide L-arginyl-glycyl-L-aspartic acid (Arg-Gly-Asp), a sequence found in the cell attachment domain of fibronectin (22–24). Small synthetic peptides containing this sequence promote cell attachment when used to coat a surface and inhibit the attachment of cells to fibronectin-coated substrata when presented in a soluble form (22, 23).

Fibronectin is not the only adhesion-promoting molecule in the immediate environs of cells. Collagens (26), laminin (5, 29), vitronectin (3, 13), and molecules such as those identified by polyclonal (6) and monoclonal (20) antibodies may also contribute to adhesion. To determine to what extent different proteins might be involved in cell adhesion, we have probed the attachment of cultured cells using the peptides that mimic the cell attachment site of fibronectin.

We report here that various cultured cell lines become detached from the substratum when grown in the presence of the active fibronectin peptides. We show also that attachment of cells to vitronectin is disrupted by the same peptides, suggesting that the mechanism(s) by which cells recognize fibronectin and vitronectin is critical for substratum attachment of cells.

MATERIALS AND METHODS

Cells and Growth Conditions: The following cell lines were cultured in Dulbecco’s modified Eagle’s medium (DME), supplemented with 5% FBS: NRK, normal rat kidney; TRK, transformed rat kidney.

Abbreviations used in this paper: DME, Dulbecco’s modified Eagle’s medium; GRGDSP, Gly-Arg-Gly-Asp-Ser-Pro; NRK, normal rat kidney; TRK, transformed rat kidney.
heat-inactivated fetal bovine serum (Tissue Culture Biologicals, Tulare, CA), glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Irvine Scientific, Santa Ana, CA); normal rat kidney (NRK); transformed rat kidney (TRK); mouse, BALB/c 3T3, NIH 3T3, Simian virus 40-transformed BALB/c 3T3; human embryonal fibroblastic (MRC-5, IMR-90, WI38); human amniotic (FL), osteosarcoma (MG-63), and fibrosarcoma (HT1080); and bovine corneal and aortic endothelial cells. Primary cultures of human umbilical cord endothelial cells were isolated by the method of Gimbrone et al. (10). They were cultured on fibronectin-coated dishes in DME, 20% fetal bovine serum, glutamine, penicillin, streptomycin, fibroblast growth factor (5 μg/ml; Collaborative Research Inc., Lexington, MA), and heparin (1 mg/ml; Sigma Chemical Co., St. Louis, MO). In some experiments, a defined serum-free medium consisting of DME supplemented with glutamine, penicillin, streptomycin, and 2 mg/ml bovine serum albumin was used to culture the cells.

**Proteins and Peptides:** Fibronectin (28) and vitronectin (13) were isolated from human plasma as previously described. Type I collagen came from Sigma Chemical Co. or (Vitrogen 100) from Flow Laboratories, Inc. (Inglewood, CA). Peptides used (Fig. 1) were synthesized to our specifications at Peninsula Laboratories, Inc. (San Carlos, CA). They were dissolved (10 mg/ml) in DME containing 0.5% bicarbonate buffer, pH 7.0, and diluted immediately for use. The peptide samples were generally used as they were received, containing 80-90% peptide, but when indicated they were first purified by high-performance liquid chromatography.

| Peptide | Activity |
|---------|----------|
| Arg - Gly - Asp - Ser | A |
| Gly - Arg - Asp - Ser - Pro | A |
| Gly - Arg - Gly - Asp - Ser - Pro - Cys | A |
| Gly - Arg - Gly - Asp - Ala - Pro | A |
| Thr - Gly - Arg - Gly | B |
| Arg - Gly - Asp - Ser - Pro - Ala - Cys | B |
| Arg - Val - Asp - Ser - Arg - Cys | B |
| Arg - Val - Asp - Ser - Thr - Ala - Cys | B |

**RESULTS**

**Disruption of Cell-Substrate Interaction by Synthetic Fibronectin Peptides**

Soluble synthetic peptides containing the cell recognition sequence Arg-Gly-Asp from fibronectin have previously been shown to interfere with the attachment of NRK cells to fibronectin-coated substrates at millimolar concentrations (23, 24). When the hexapeptide Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) was added to routine cultures of NRK cells at a concentration of 1 mg/ml (1.5 mM), the cells became detached from the substrate (Fig. 2a). The effect appeared to be
specific, as a control peptide that contained an Asp-to-Glu substitution and lacks cell attachment activity (24) had no noticeable effect on the cells at the same concentration (Fig. 2b).

Experiments carried out under various conditions showed that the detaching effect of the active peptide was most readily demonstrable when cells were plated 24 h before the addition of the peptide at a density that gave a 70–90% confluent culture when the peptide was added. The peptide was also effective in detaching older and denser cultures, but the effects were somewhat less pronounced. However, detachment efficiency was increased in all cases if serum was removed before the peptide was added.

When the GRGDSP peptide was added to cultures of NRK cells under the conditions established as most favorable for the detachment (24 h culture with the peptides added in serum-free medium), the cells began to round up within 30 min (Fig. 3a). Holes subsequently appeared in the monolayer as the cells retracted into ridges (Fig. 3b). After a few hours, the cells completely detached from the culture plates, forming floating aggregates (Fig. 3c). Cells cultured in the presence of a control peptide (as shown for the tetrapeptide Thr-Gly-Arg-Gly in Fig. 3d) were unaffected even after prolonged exposure to the peptide.

Quantitation of Peptide-induced Detachment in Different Cell Types

Titration of peptides in NRK cell cultures followed by counting of the remaining attached cells showed that the GRGDSP peptide was effective in detaching these cells at and above 10 μg/ml (Fig. 4, top left). The activities of the crude and high-performance liquid chromatography-purified GRGDSP peptide were similar. The detachment of the cells was

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**Figure 3** Time course of cell detachment by Arg-Gly-Asp-containing peptides. NRK cells were cultured for 24 h on tissue culture dishes in the presence of serum, and the culture medium was removed and replaced with serum-free medium containing 1 mg/ml GRGDSP peptide. The cells were photographed 30 min (a), 1.5 h (b), and 3 h (c) after the addition of the peptide. No detachment was obtained with a control peptide (Thr-Gly-Arg-Gly 1 mg/ml) even at 16 h (d). × 120.
Attachment to Collagen

To study the correlation of detaching capacity with the cell types. A number of cell types in the presence of the peptides. The Arg-Gly-Asp-containing peptides, which have attachment-promoting activity, detached the cultured NRK cells. In contrast, the five peptides that lack this sequence and do not promote cell attachment could not detach the NRK cells.

To determine whether the fibronectin peptides would cause detachment of cells other than the NRK cells, we incubated a number of cell types in the presence of the peptides. The Arg-Gly-Asp-containing peptides were effective in detaching all of the 13 cell strains tested. Examples of the peptide-mediated detachment on these other cell lines are shown in Fig. 4, and a complete list of the cells tested is given in Materials and Methods. Represented among these lines are normal and transformed cells, established cell lines and primary cultures, fibroblastic, endothelial, and epithelial cell types.

Inhibition of Cell Attachment to Vitronectin by the Fibronectin Peptides and Lack of Effect on Attachment to Collagen

Fibronectin is not the only adhesion-promoting molecule found in the extracellular matrix of cells in culture (19). In particular these matrices are rich in different types of collagen, which have been shown to promote cell attachment (26) and which contains Arg-Gly-Asp sequences (24). Moreover, vitronectin from the cells, or from the culture medium when the cells are cultured in the presence of serum (13), could be involved in the substratum adhesion of the cells. It was therefore of interest to see whether the Arg-Gly-Asp-containing peptides would also affect the attachment of cells to collagen and vitronectin. Previous work has shown that cell attachment mediated by laminin, another adhesive extracellular matrix protein, does not depend on this mechanism (17, 25).

An assay for inhibition of cell attachment similar to the one previously used to show that Arg-Gly-Asp-containing peptides can inhibit attachment of cells to fibronectin (23) was used to study attachment to vitronectin and collagen. We found that the GRGDSP and other Arg-Gly-Asp-containing peptides prevented attachment of the NRK test cells to microtiter wells coated with vitronectin at concentrations similar to those that inhibited attachment to fibronectin (Fig. 5). In contrast, the peptides were incapable of preventing the attachment of the cells to wells coated with type I collagen. Titration of the collagen used to coat the dishes showed that the GRGDSP peptide had no effect on the attachment of the NRK cells to such dishes, even at the lowest collagen concentrations (Fig. 6). Similar results were obtained with the human IMR-90 and MRC-5 embryonal fibroblasts (not shown).

Presence of Collagens in the Extracellular Matrix of Cells Sensitive to Peptide Detachment

Since we could not prevent the attachment of cells to surfaces coated with collagen, but the Arg-Gly-Asp-containing peptides detached the same cells in culture, it seemed either that collagen was not involved in the attachment of these cells or that they might not have collagen in their extracellular matrix under the conditions used in this study. We therefore examined the matrix of one such cell line for the presence of collagen. The human IMR-90 fibroblasts were used for these experiments to allow detection of collagens with antibodies prepared against human collagens. In agreement with earlier studies on human fibroblasts (8, 30), type I collagen was readily detected at the cell surface and in the extracellular matrix.
different amounts of type I collagen, and NRK cells were plated or buffer only (○). Cell attachment was measured as described in the legend of Fig. 4.

Sence of GRGDSP peptide. Microtiter wells were coated into the wells in the presence of 1 mg/ml of GRGDSP peptide (○) or buffer only (○). Cell attachment was measured as described in the legend of Fig. 4.

FIGURE 6 Attachment of cells to collagen in the presence or absence of GRGDSP peptide. Microtiter wells were coated with different amounts of type I collagen, and NRK cells were plated into the wells in the presence of 1 mg/ml of GRGDSP peptide (○) or buffer only (○). Cell attachment was measured as described in the legend of Fig. 4.

extracellular matrix of the IMR-90 cells by immunofluorescent staining (Fig. 7a). Moreover, it is known that the type I collagen is present in the same matrix structures as fibronectin because the two proteins codistribute in immunofluorescent staining (30). A similar co-distribution with fibronectin is shown in Fig. 7 (b and c) for type VI collagen, which is also a cell attachment-promoting protein (E. Engvall, personal communication). Thus, the lack of extracellular matrix collagen is not the reason for the sensitivity of cells to the detaching effects of the peptides. However, since the secretion and processing of collagens is increased by ascorbic acid, we also tested fibroblasts grown in the presence of 50 μg/ml ascorbic acid for their response to the peptides. It was found that cells grown in this manner were fully susceptible to detachment with the GRGDSP peptide (result not shown).

Detachment of Cells Cultured on Collagen in the Presence and Absence of Serum

As shown above, cells newly seeded on collagen-coated dishes are refractile to the detaching effect of the Arg-Gly-Asp peptides. To determine whether such cells would remain resistant to the peptides, we studied detachment of cells after different periods of culture on collagen. Such cells became susceptible to the peptides after 24 h in culture (results not shown). This acquired susceptibility to the peptides could be due to the cells switching their attachment from collagen to fibronectin synthesized by the cells themselves. However, since the cells were cultured in the presence of serum, they also might be using fibronectin or vitronectin acquired from the serum and would, because of this, be sensitive to detachment by the Arg-Gly-Asp peptides. Serum vitronectin in particular could be involved because our recent observations show that most of the cell attachment-promoting activity in fetal bovine serum is associated with vitronectin (our unpublished results).

To gain information about the possible involvement of serum, we studied detachment of the NRK cells and TRK cells seeded and cultured on type I collagen in serum-free medium. Such cells also became susceptible to the detaching effect of the GRGDSP peptide after 24 to 36 h in culture (Fig. 8). Serum, therefore, is not required for these cells to replace their interaction with the collagen with an Arg-Gly-Asp-dependent attachment mechanism.

Effect of Peptide-induced Detachment on Cell Growth

Since anchorage-dependent cells usually cannot proliferate if denied substrate attachment (2, 11), it was of interest to determine the effect of the peptide treatment on cell growth. When NRK cells were cultured in the presence of 1% serum and concentrations of the GRGDSP peptide that detached the cells, the number of live cells remained stationary (Fig. 9). In contrast, in the presence of a control peptide or medium alone, cell number increased with a doubling time of 36 h.

DISCUSSION

We show here that peptides containing the sequence Arg-Gly-Asp, the minimum sequence recognized by cells in fibronectin (23, 24), can cause detachment of cultured cells. The detaching effect of the Arg-Gly-Asp peptides is specific and not due to toxicity. The specificity was demonstrated by the fact that control peptides synthesized by the same procedures but that lacked the Arg-Gly-Asp sequence had no observable effect on cells. Lack of toxicity was evidenced by the observation that no cell death was detected in the cultures detached with the active peptides by the trypan blue exclusion test, and that the detached cells reattached and grew after removal of the peptide.

Sensitivity toward the fibronectin peptides appears to be widespread among different types of cells and it is manifested under a variety of culture conditions. The peptides were effective on human, rat, bovine, and mouse cells representing fibroblastic, endothelial, and epithelial cell types both in primary culture and as established cell lines. Moreover, the peptides were effective in detaching cells that organize an elaborate extracellular matrix, e.g., the NRK cells, as well as on cells that lack such a matrix, e.g., TRK cells (12). We observed the effect whether or not the cells were plated in the presence of serum. Although our observations have all been made using cells cultured in vitro, the diversity of the cell types and culture conditions in which the Arg-Gly-Asp peptides detach cells suggests that this mechanism is intrinsic to the cells and therefore probably significant also in vivo. In fact, Boucaut et al. (4) recently reported that a synthetic peptide that contains an Arg-Gly-Asp sequence prevented gastrulation when injected into amphibian embryos.

Since the detached cells assembled into aggregates, it appears that the fibronectin peptides interfered primarily with cell-substratum adhesion but not with cell-cell adhesion, indicating that their cell-cell interaction mechanisms remained unaffected by the peptides. This agrees with previous observations that assign cell-cell adhesion to molecules different from those known to be involved in cell-substratum interaction (7, 18).

It is not clear at this time which molecules mediate the cell-substratum interactions perturbed by the Arg-Gly-Asp peptides. Fibronectin is probably involved, since the peptides inhibit and reverse cell attachment to it and since it is abundant in the extracellular matrix of most cultured cells. Our results show that the attachment of cells to vitronectin is also
prevented and reversed by the Arg-Gly-Asp-containing peptides. Since there are substantial quantities of vitronectin in serum and, judging from immunofluorescence with monoclonal antibodies, it also occurs at cell surfaces (13), vitronectin probably mediates at least some of the cell-substrate adhesions affected by the peptides. Whether vitronectin also has the Arg-Gly-Asp recognition sequence in its cell attachment site remains to be determined.

Collagens are rich in Arg-Gly-Asp sequences, but the significance of these sequences to the cell attachment properties of collagen is not known (23). The Arg-Gly-Asp peptides did not inhibit the attachment of cells to collagen, suggesting that in collagen the Arg-Gly-Asp sequences may not be the main recognition sites for cells. The lack of inhibition could also be interpreted as a failure to achieve a high enough concentration of the peptide at the multiple Arg-Gly-Asp sites that occur in collagen (24). However, since the various cultured cells were readily detached from their matrices by the Arg-Gly-Asp-containing peptides, our results suggest that collagens, which our immunological data show to be present in the matrices of the cells studied, do not participate significantly in the cell-substratum interactions. Attachment mechanisms other than those dependent on the Arg-Gly-Asp sequence may operate transiently but become less important as cells become established on a matrix. Peptides that duplicate some of the most common sequences in collagen such as (Gly-Pro-Pro)$_n$ have been found to promote the attachment of hepatocytes, albeit only weakly (26).

Since the Arg-Gly-Asp peptides mimic the cell attachment site in fibronectin (and apparently also in vitronectin), it seems likely that they detach the cultured cells by competing for attachment receptors at the cell surface. A cell surface receptor that recognizes the Arg-Gly-Asp sequence was recently identified in MG-63 human osteosarcoma cells and in the NRK cells (25). The possibility remains, however, that the interaction of the peptides with such a receptor could cause detachment of the cells by a mechanism that does not involve competition with substratum-bound molecules. The synthesis of proteases (1) or reorganization of the cytoskeleton (15) are examples of such possible mechanisms. However, if such

**Figure 7** Presence of fibronectin and collagen in IMR-90 extracellular matrix. Immunofluorescent staining of a subconfluent 24-h IMR-90 cell culture for collagen type I (a) and a double staining of a similar culture for type VI collagen (b) and fibronectin (c). The type VI collagen fluorescence was detected with fluorescein-conjugated anti-rabbit IgG and fibronectin with rhodamine-conjugated anti-mouse IgG. (d) Phase-contrast photograph of the same cells as in b and c. × 320.
mechanisms were involved, they would have to be specifically triggered by the cell attachment receptors, because only the Arg-Gly-Asp peptides have the detachment-promoting effect.

NRK cells completely detached from the substrate with the Arg-Gly-Asp peptides were arrested in their growth. This is in agreement with the well known fact that cells denied substrate anchorage are not able to grow (2, 11). It is interesting that some cells (e.g., IMR-90, Fig. 4) were more susceptible to the detaching effects of the Arg-Gly-Asp peptides than others (e.g., FL cells), suggesting that the synthetic peptides might allow manipulation of cell attachment and growth with some degree of selectivity. It will also be interesting to see whether peptides capable of detaching cells via the same mechanism might occur physiologically. Such peptides could play a role in loosening cells from the substrate during cell division and migration.

Finally, there may be practical applications for the observations described in this paper, because the Arg-Gly-Asp peptides appear to provide a gentle method for the detachment of cells from culture plates for subculturing and experimentation. That it might be possible to use this method to detach cells from tissues is an even more promising possibility because the standard protease treatments often are not gentle enough for this purpose.

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