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Analysis of $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins in cell-collagen interactions: identification of conformation dependent $\alpha_1\beta_1$ binding sites in collagen type I

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Integrins can mediate the attachment of cells to collagen type I. In the present study we have investigated the possible differences in collagen type I recognition sites for the $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins. Different cyanogen bromide (CB) fragments of the α1(I) collagen chain were used in cell attachment experiments with three rat cell types, defined with regard to expression of collagen binding integrins. Primary rat hepatocytes expressed $\alpha_1\beta_1$, primary rat cardiac fibroblasts $\alpha_1\beta_1$ and $\alpha_2\beta_1$, and Rat-1 cells only $\alpha_2\beta_1$. All three cell types expressed $\alpha_3\beta_1$ but this integrin did not bind to collagen-Sepharose or to immobilized collagen type I in a radioreceptor assay. Hepatocytes and cardiac fibroblasts attached to substrata coated with α1(I)CB3 and α1(I)CB8; Rat-1 cells attached to α1(I)CB3 but only poorly to α1(I)CB8-coated substrata. Cardiac fibroblasts and Rat-1 cells spread and formed β_1-integrin-containing focal adhesions when grown on substrata coated with native collagen or α1(I)CB3; focal adhesions were also detected in cardiac fibroblasts cultured on α1(I)CB8. The rat α_1 specific monoclonal antibody 3A3 completely inhibited hepatocyte attachment to α1(I)CB3 and α1(I)CB8, as well as the attachment of cardiac fibroblasts to α1(I)CB8, but only partially inhibited the attachment of cardiac fibroblasts to α1(I)CB3. 3A3 IgG did not inhibit the attachment of Rat-1 cells to collagen type I or to α1(I)CB3. These data indicate that binding sites for $\alpha_1\beta_1$ are present in both α1(I)CB3 and α1(I)CB8 and that α1(I)CB3, but not α1(I)CB8, contains a binding site for $\alpha_2\beta_1$, and suggest that collagen type I contains separate binding sites for $\alpha_1\beta_1$ and $\alpha_2\beta_1$. Hepatocyte attachment to heat-denatured collagen type I was inhibited by the hexapeptide GRGDTP. It therefore appears that denaturation both destroys the integrity of conformation dependent binding sites for $\alpha_1\beta_1$ and reveals a cryptic RGD-containing site recognized by the $\alpha_5\beta_1$ of hepatocytes.

Key words: binding sites/β_1 integrin/collagen type I

Introduction

Interaction of cells with molecules of the extracellular matrix (ECM) has been shown to influence many cellular activities. Prominent among ECM components are large, extended, glycoproteins composed of multiple, structurally diverse domains. For many such ECM proteins it is now clear that cells may interact with two or more distinct regions within the same macromolecule. To understand the molecular mechanisms responsible for ECM modulation of cellular functions, it is thus essential to identify and characterize the receptor binding sites within the different ECM molecules and in turn the specific receptors that bind to these sites.

Members of the integrin family of cell adhesion molecules act as receptors for a number of extracellular ligands (Hynes, 1987, 1992; Ruoslahti and Pierschbacher, 1987; Albelda and Buck, 1990). Integrins are $\alpha\beta$ heterodimeric transmembrane glycoproteins that have been classified into at least eight subfamilies, designated $\beta_1-\beta_8$ (reviewed in Hynes, 1992). Within each subfamily, the same β subunit can associate with one of a set of different α subunits. Each heterodimer has its own ligand binding selectivity, with many integrins binding to more than one ECM protein. Certain α subunits can also associate with multiple β chains (reviewed in Hynes, 1992).

The first integrin binding site to be identified was the RGD-containing sequence in fibronectin (Pierschbacher and Ruoslahti, 1984). The only β_1 integrin that binds with high affinity to this site in fibronectin is $\alpha_5\beta_1$ (Pytela *et al.*, 1985; Takada *et al.*, 1987). Primary rat hepatocytes express the $\alpha_5\beta_1$ integrin that functions as a high affinity fibronectin receptor on these cells (Forsberg *et al.*, 1990). RGD-containing sequences in other proteins, such as vitronectin, von Willebrand factor, thrombospondin and osteopontin, also support cell adhesion, but in these cases the integrins responsible for mediating RGD dependent adhesion are mainly β_3 and β_5 integrins (Ginsberg *et al.*, 1988; Lawler *et al.*, 1988; Oldberg *et al.*, 1988; McLean *et al.*, 1990). RGD-independent binding to fibronectin has also been described. The IIICS region, present in some forms of fibronectin as a result of alternative splicing, is recognized by the integrin $\alpha_4\beta_1$ (Wayner *et al.*, 1989).

For another ECM protein, laminin, at least two distinct and spatially well separated binding sites have been shown to be recognized by various β_1 integrins. It appears that $\alpha_3\beta_1$ and $\alpha_6\beta_1$ bind to site(s) near the end of the long arm of the cruciform laminin, while $\alpha_1\beta_1$ recognizes a site near the cross region (Gehlsen *et al.*, 1989; Aumailley *et al.*, 1990; Forsberg *et al.*, 1990; Hall *et al.*, 1990; Sonnenberg *et al.*, 1990; Tomaselli *et al.*, 1990). Of these integrins, $\alpha_1\beta_1$ is a receptor for collagens (including types I, II and IV) as well as for laminin (Kramer and Marks, 1989; Turner *et al.*, 1989; Forsberg *et al.*, 1990; Gullberg *et al.*, 1990), $\alpha_6\beta_1$ acts as a receptor for laminin (Sonnenberg *et al.*, 1990), while $\alpha_3\beta_1$ is reported to have affinity for

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collagen and fibronectin in addition to laminin (Wayner and Carter, 1987).

There are thus multiple integrins with selectivity for defined regions of ECM components which themselves can vary in structure. This suggests that the effects of a given matrix molecule may be mediated by distinct ligand–receptor systems acting via distinct intracellular signalling mechanisms. In general, the response of a cell to a particular form of an ECM molecule will depend on the number and subunit composition of the integrins expressed on its surface and their specific ligand binding abilities.

In our earlier studies of β_1-integrin-mediated adhesion of primary cells to collagen type I we found that anti-β_1 integrin IgG inhibited hepatocyte adhesion to the α1(I)CB3 fragment, which lacks the RGD sequence (Gullberg *et al.*, 1989). The major collagen binding integrin on rat hepatocytes, $\alpha_1\beta_1$, could be isolated by affinity chromatography on columns derivatized with α1(I)CB3 (Gullberg *et al.*, 1990). From primary fibroblasts, on the other hand, we isolated two integrins that bound to α1(I)CB3: $\alpha_1\beta_1$ and a heterodimer with characteristics of $\alpha_2\beta_1$. Staatz *et al.* (1991) have shown that $\alpha_2\beta_1$ from platelets recognizes the sequence DGEA present in α1(I)CB3. A major conformation dependent recognition site for $\alpha_1\beta_1$ and $\alpha_2\beta_1$ was recently localized to the CB3 fragment of collagen type IV (Vandenberg *et al.*, 1991). In addition, the multifunctional $\alpha_3\beta_1$ integrin has been reported to mediate cell attachment to collagen (Wayner and Carter, 1987; Takada *et al.*, 1988). In this report we wanted to assess the involvement of these integrins in collagen attachment using cells with differing combinations of these collagen binding integrins.

Results

Expression of collagen type I binding β_1 integrins on hepatocytes, cardiac fibroblasts and Rat-1 cells

Primary rat hepatocytes and cardiac fibroblasts both express on their surfaces the integrin $\alpha_1\beta_1$ that can function as a collagen receptor (Gullberg *et al.*, 1990). The expression of β_1 integrins by cardiac fibroblasts and Rat-1 cells was compared by immunoprecipitation of extracts from ^{125}I-labeled cells (Figure 1). In separate experiments the α_1, α_2 and α_3 chains were identified by means of immunoprecipitation using the various α chain specific antibodies (see Materials and methods); the positions of these α chains are indicated in Figure 1. The most evident difference between the Rat-1 cells and the cardiac fibroblasts was that the α_1 chain appeared to be absent from Rat-1 cells, but was easily detected on the cardiac fibroblasts (Figure 1). Both Rat-1 cells and cardiac fibroblasts expressed α_2 chains on their cell surfaces; the α_2 chain from Rat-1 cells migrated as a M_r 140 000 protein, while that from cardiac fibroblasts migrated as a M_r 150 000 protein; the M_r 135 000 α_3 chain was present in both Rat-1 cells and primary fibroblasts (Figure 1).

Affinity chromatography on Sepharose-4B derivatized with collagen type I was used to identify collagen binding integrins in extracts from the three different types of cells. In accordance with earlier findings (Gullberg *et al.*, 1990), proteins with M_rs 180 000, 150 000 and 115 000 were purified from extracts of ^{125}I surface-labeled cardiac fibroblasts (Figure 2, lane a). From extracts of primary rat hepatocytes only the M_r 180 000/150 000 heterodimer,

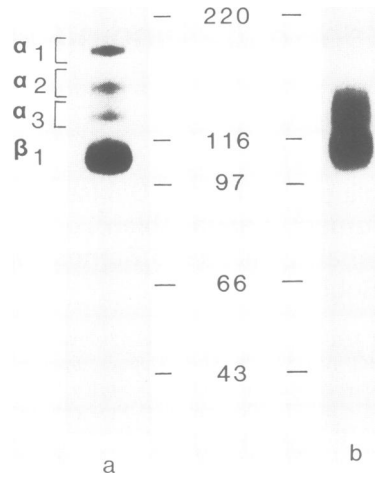

Fig. 1. Analysis of surface expression of β_1 integrins on primary rat cardiac fibroblasts and Rat-1 cells. Detergent solubilized proteins from surface-iodinated cardiac fibroblasts (**a**) or Rat-1 cells (**b**) were subjected to immunoprecipitation with anti-β_1 integrin IgG. Precipitated proteins were electrophoresed under non-reducing conditions on 5–10% SDS–polyacrylamide gels followed by autoradiography. The identity of the α chains was determined by immunoprecipitations with α chain specific antibodies in separate experiments.

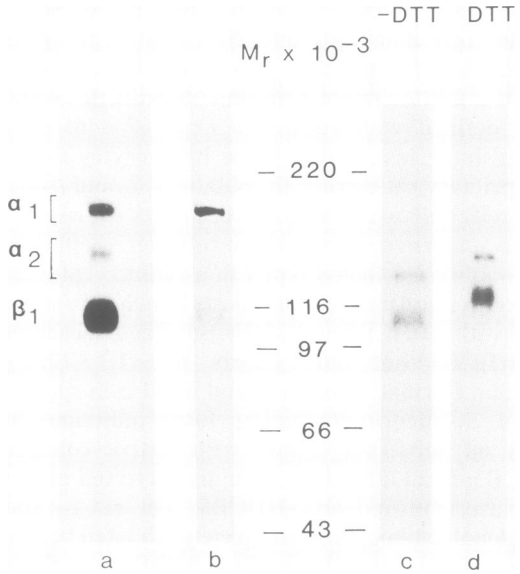

Fig. 2. Comparison of collagen binding integrins from hepatocytes, cardiac fibroblasts and Rat-1 cells. Detergent extracts from cardiac fibroblasts (lane a), hepatocytes (lane b) and Rat-1 cells (lanes c and d) were subjected to affinity chromatography on collagen type I–Sepharose as described in Materials and methods. Purified proteins were electrophoresed on 5–10% SDS–polyacrylamide gels under non-reducing (lanes a–c) or reducing (lane d) conditions and visualized by silver staining (lane b) or autoradiography (lanes a, c and d). All protein bands in lanes a–d were specifically immunoprecipitable with anti-β_1 integrin IgG (data not shown).

previously shown to be the $\alpha_1\beta_1$ integrin, bound to collagen–Sepharose (Figure 2, lane b). From surface-iodinated Rat-1 cells, two proteins with M_rs of 140 000 and 115 000 were recovered (Figure 2, lane c). The M_r 140 000 protein from Rat-1 cells migrated as an M_r 150 000 protein after reduction (Figure 2, lane d) and with

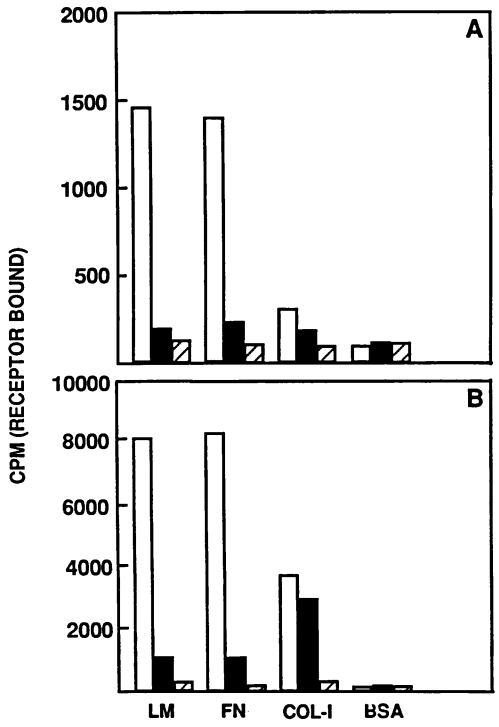

Fig. 3. Binding of ^{125}I-labeled laminin binding integrins to immobilized matrix components. Laminin binding integrins were isolated and iodinated as detailed in Materials and methods. Binding of laminin binding integrins from Rat-1 cells (**A**) and from cardiac fibroblasts (**B**) to immobilized laminin (LM); human plasma fibronectin (FN); collagen type I (COL-1); and to bovine serum albumin (BSA) was quantified as described in Materials and methods. The binding experiments were performed in PBS (open bars); in PBS with 50 μg/ml anti-rat α_3 IgG (filled bars); and in PBS with 50 μg/ml anti-β_1 IgG (hatched bars). Laminin binding proteins from cardiac fibroblasts were labeled to a higher specific activity than proteins from Rat-1 cells. The data are from two separate experimental series that gave qualitatively identical results.

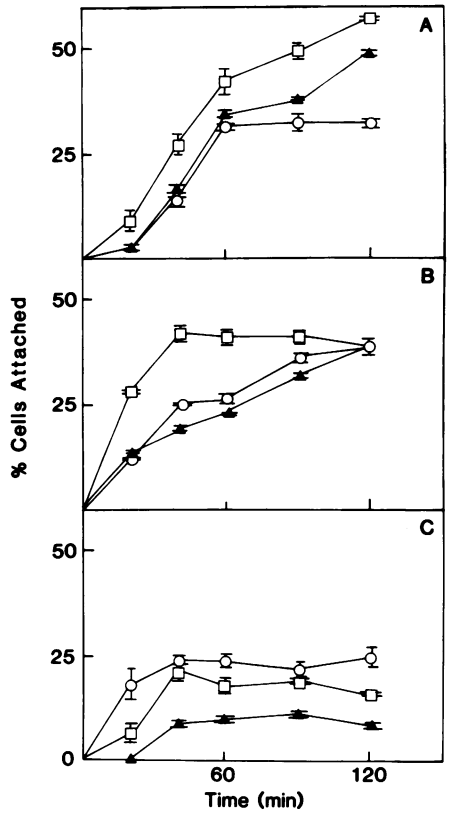

Fig. 4. Cell attachment to collagen type I, collagen fragments α1(I)CB3 and α1(I)CB8. Hepatocytes (**A**), cardiac fibroblasts (**B**) and Rat-1 cells (**C**) were allowed to attach at 37°C to dishes coated with collagen type I (□--□); α1(I)CB3 (○--○); and α1(I)CB8 (▲--▲) for the indicated time periods. Vertical bars represent the total range of duplicate dishes.

the same M_r as the protein immunoprecipitated by α_2 chain specific IgG from extracts of these cells (data not shown). The M_r 115 000 protein present in the collagen type I–Sepharose eluates from extracts of all three types of cells could be specifically immunoprecipitated by anti-β_1 integrin IgG (data not shown). Prolonged exposure of the Rat-1-derived material eluted from collagen type I–Sepharose revealed traces of a protein band with an M_r of 180 000, indicating the presence of low amounts of the $\alpha_1\beta_1$ integrin (data not shown). Immunoprecipitation with α specific antibodies of integrins in extracts of surface-iodinated hepatocytes was difficult to achieve due to the low incorporation of ^{125}I into surface proteins of these cells. However, a M_r 150 000 protein was detected in Triton X-100 extracts of hepatocytes by immunoblotting with the anti-α_3(pep) IgG (data not shown). The integrin $\alpha_3\beta_1$ is thus expressed in all three types used.

Rat fibroblast $\alpha_3\beta_1$ does not bind to collagen type I

The integrin $\alpha_3\beta_1$ has been reported to have affinity for collagen (see Introduction), yet $\alpha_3\beta_1$ was not detected in collagen type I–Sepharose eluates derived from extracts of any of the three reported types of cells studied (Figure 2). To investigate further the potential binding of $\alpha_3\beta_1$ to collagen, laminin binding integrins were purified from cardiac fibroblasts and Rat-1 cells, respectively, by means of affinity chromatography on human placental laminin; this material was expected to be enriched in $\alpha_3\beta_1$ (Gehlsen *et al.*, 1988). In the purified and iodinated material from Rat-1 cells, $\alpha_3\beta_1$ was the only integrin detected (Materials and methods). Iodinated laminin binding material from the two sources bound readily to fibronectin and laminin immobilized on microtiter plates; binding was inhibited by both anti-β_1 integrin IgG and anti-rat α_3 antibodies (Figure 3A and B). The iodinated material from Rat-1 cells, which lacked detectable $\alpha_1\beta_1$ or $\alpha_2\beta_1$, bound poorly to collagen type I (Figure 3A). By contrast, the labeled material from cardiac fibroblasts bound to collagen type I (Figure 3B), and this binding was inhibited by anti-β_1 integrin IgG but not by anti-rat α_3 IgG (Figure 3A and B).

Cell attachment to collagen type I and collagen type I fragments

The extent and kinetics of attachment of hepatocytes, cardiac fibroblasts and Rat-1 cells to native collagen type I, collagen fragment α1(I)CB3 and collagen fragment α1(I)CB8 were investigated. Hepatocytes and cardiac fibroblasts adhered effectively to all three substrata (Figure 4A and B). Rat-1 cells adhered to a lesser degree; attachment to α1(I)CB8 was particularly low and demonstrated a marked lag phase (Figure 4C).

The 3A3 antibody that specifically recognizes rat $\alpha_1\beta_1$ and inhibits its function (Turner *et al.*, 1989; Tawil *et al.*,

1990; Tomaselli *et al.*, 1990) was earlier shown to inhibit hepatocyte attachment to collagen type I and partly to inhibit primary fibroblast attachment to collagen type I (Gullberg *et al.*, 1990). Attachment of hepatocytes to α1(I)CB3 and α1(I)CB8, as well as to collagen type I, was completely inhibited by 3A3 IgG (Figure 5A). 3A3 IgG only partially inhibited attachment of cardiac fibroblasts to collagen type I or α1(I)CB3; however, the attachment of these cells to α1(I)CB8 was completely inhibited by the antibody (Figure 5B). 3A3 IgG had no effect on Rat-1 cell attachment to native collagen type I or to α1(I)CB3 (Figure 5C).

Rat-1 cells and cardiac fibroblasts cultured on native collagen type I and collagen fragments for 4 h displayed marked differences with regard to the organization of integrins and actin stress fibers. The cardiac fibroblasts exhibited more β_1-integrin-containing focal contacts, as well as a more extensive actin cytoskeleton than the Rat-1 cells (Figures 6 and 7). β_1-integrin-containing focal contacts were formed by cardiac fibroblasts cultured on native collagen type I and on α1(I)CB3 and α1(I)CB8 (Figure 6A, B and C). Rat-1 cells similarly formed β_1-integrin-containing focal contacts when grown on native collagen type I and on α1(I)CB3 (Figure 7A and B). The $\alpha_1\beta_1$ integrin was present in focal contacts on the primary fibroblasts on all three substrata (Figure 6D, E and F), whereas as expected, the $\alpha_1\beta_1$ integrin could not be visualized on Rat-1 cells (Figure 7C and D). Stress fibers were present in both types of cells, although less abundantly and not as elaborately organized in Rat-1 cells (Figure 7E and F) as in cardiac fibroblasts (Figure 6G, H and I).

The attachment of hepatocytes to native collagen and to α1(I)CB8 was not inhibited by the synthetic hexapeptide GRGDTP; however, the attachment of hepatocytes to fibronectin and the small extent of attachment of these cells to denatured collagen coated at 37°C were effectively inhibited by this peptide (Figure 8).

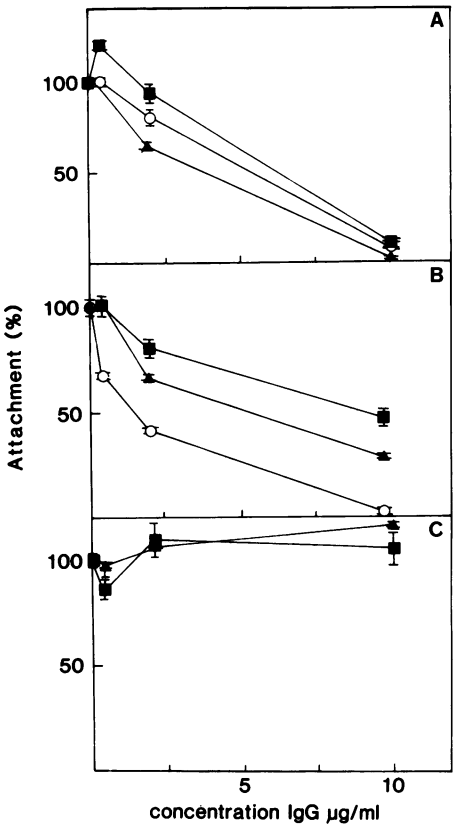

Fig. 5. Inhibition of cell attachment to collagen type I, collagen fragments α1(I)CB3 and α1(I)CB8 by antibody specific for $\alpha_1\beta_1$. Hepatocytes (**A**), cardiac fibroblasts (**B**) and Rat-1 cells (**C**) were seeded into culture wells coated with collagen type I (■--■), α1(I)CB3 (▲--▲) and α1(I)CB8 (○--○) in the presence of the indicated amounts of 3A3 IgG. Cells were allowed to attach at 37°C for 40 min. Data are expressed as percentage of cell attachment in the absence of antibody. Vertical bars indicate the range of duplicate incubations.

Discussion

Previous work had shown that the integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ can both function as collagen receptors (Wayner and Carter, 1987; Kramer and Marks, 1989; Takada *et al.*, 1988; Turner *et al.*, 1989; Staatz *et al.*, 1990, 1991; Gullberg *et al.*, 1990) and that both recognize a site present in the α1(I)CB3 fragment of collagen type I (Gullberg *et al.*, 1990; Staatz *et al.*, 1990); evidence has recently been presented that $\alpha_2\beta_1$ recognizes the sequence Asp-Gly-Glu-Ala in the α1(I)CB3 fragment of collagen type I (Staatz *et al.*, 1991). In the present study we set out to determine whether $\alpha_1\beta_1$ and $\alpha_2\beta_1$ bind to the same or different sites in α1(I)CB3, using rat cells that express $\alpha_1\beta_1$ but not $\alpha_2\beta_2$ (hepatocytes), $\alpha_2\beta_1$ but not $\alpha_1\beta_1$ (Rat-1 cells), or both $\alpha_1\beta_1$ and $\alpha_2\beta_1$ (cardiac fibroblasts).

Before we could proceed, however, we had to deal with a potentially complicating factor: the possibility that one or more of the cell types also expressed $\alpha_3\beta_1$ on their surfaces. Two lines of evidence have been taken as indicating that human $\alpha_3\beta_1$ can function as a collagen attachment receptor: (i) $\alpha_3\beta_1$ from HT1080 cells can be isolated by affinity chromatography on collagen type I– and VI–Sepharose under conditions that allow for low-affinity binding (Wayner and Carter, 1987); and (ii) antibodies that block the function of $\alpha_3\beta_1$ partially inhibit the adhesion of HT1080 cells to collagen-coated substrata (Takada *et al.*, 1988). Both Rat-1 cells and cardiac fibroblasts were readily shown to express $\alpha_3\beta_1$ (Figures 1 and 3) and hepatocytes contain $\alpha_3\beta_1$ detectable by immunoblotting (data not shown). Inasmuch as all three types of cells analyzed in the present report expressed $\alpha_3\beta_1$, it was important to investigate the possible involvement of $\alpha_3\beta_1$ in attachment to collagen.

No binding of $\alpha_3\beta_1$ from any of the three cell types to collagen type I–Sepharose was seen (Figure 2). Furthermore, ^{125}I-labeled $\alpha_3\beta_1$ purified from Rat-1 cells displayed a very low binding to collagen type I in a radioreceptor assay (Figure 3A). A preparation enriched in $\alpha_3\beta_1$, purified by laminin affinity chromatography from extracts of cardiac fibroblasts, bound to collagen type I (Figure 3B); the binding was inhibited by anti-β_1 integrin IgG but not by anti-rat α_3 IgG (Figure 3B). Since cardiac fibroblasts contain $\alpha_1\beta_1$ and since $\alpha_1\beta_1$ has affinity for laminin (Turner *et al.*, 1989; Forsberg *et al.*, 1990), the latter result can probably be explained by the presence of functionally significant amounts of $\alpha_1\beta_1$ in addition to $\alpha_3\beta_1$. Collectively our results suggest that solubilized $\alpha_3\beta_1$ isolated from different rat cells has little or no affinity for collagen type I. Furthermore, from attachment experiments using the 3A3 antibody it can be concluded that $\alpha_3\beta_1$ did not mediate the attachment of hepatocytes to collagen type I, or to the collagen fragments

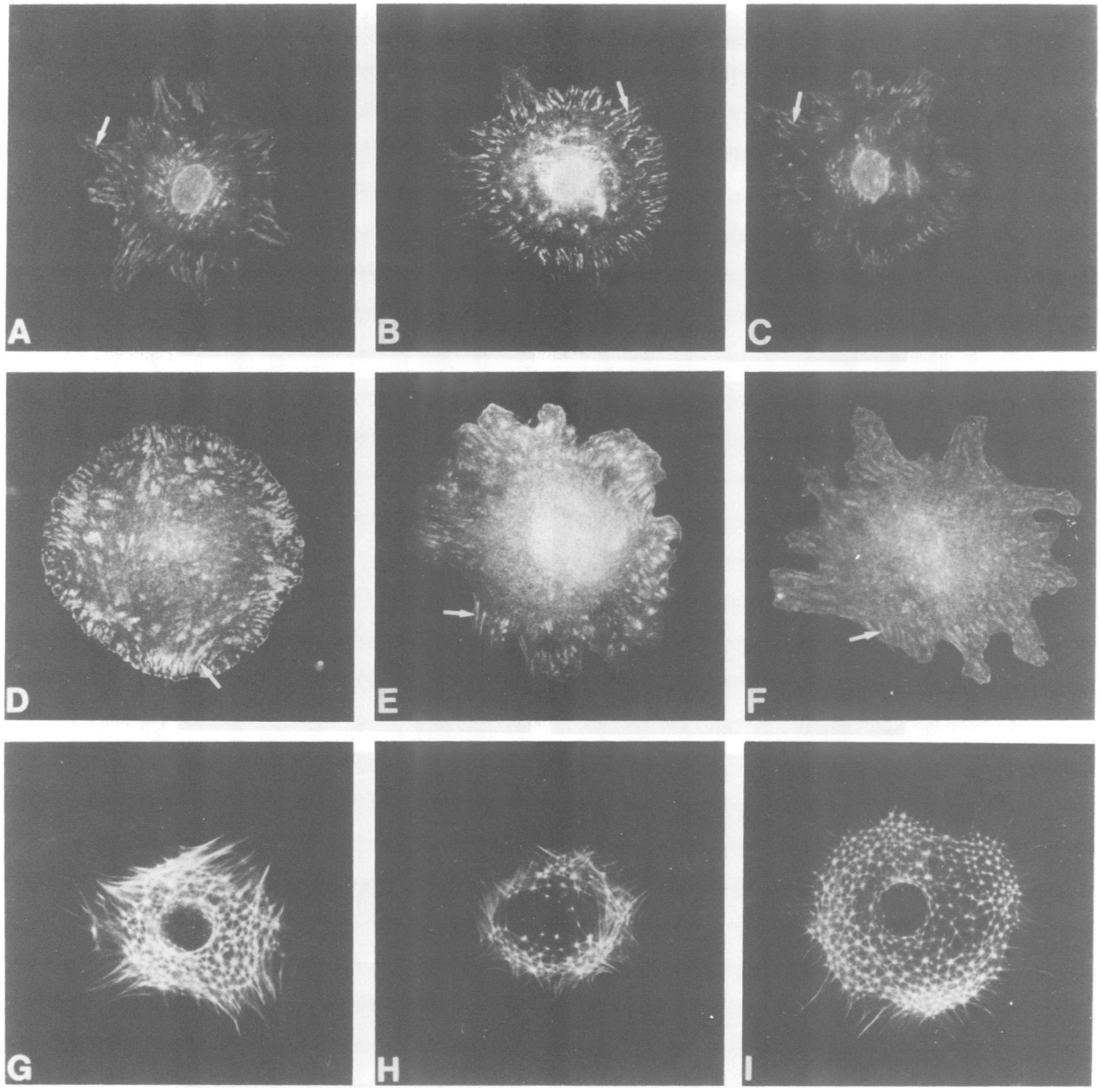

Fig. 6. Distribution of F-actin, the β_1 and $\alpha_1\beta_1$ integrin chains on primary cardiac fibroblasts cultured on collagenous substrata. Cells were seeded on coverslips coated with collagen type I (**A**, **D** and **G**), collagen fragment α1(I)CB3 (**B**, **E** and **H**) or collagen fragment α1(I)CB8 (**C**, **F** and **I**) and cultured for 4 h in serum-free media as described in Materials and methods. Cells were stained by indirect immunofluorescence for the β_1 integrin chain (A–C); for the $\alpha_1\beta_1$ integrin (D–F); or by phalloidin staining for F-actin (G–I). Magnification ×245.

α1(I)CB3 or α1(I)CB8, or the attachment of cardiac fibroblasts to α1(I)CB8 (see below). Also, anti-rat α_3 IgG did not inhibit attachment of Rat-1 cells or cardiac fibroblasts to collagen, but inhibited the attachment of these cells to laminin (data not shown). This cell behavior confirms the findings with solubilized $\alpha_3\beta_1$.

Hepatocytes bind well to intact collagen type I and to two fragments derived from the main triple-helical segment of the collagen type I molecule (Figure 4A). The complete inhibition of hepatocyte attachment to collagen type I and to both fragments by the monoclonal antibody, 3A3 (Figure 5A), confirms that $\alpha_1\beta_1$ is the only functionally significant collagen binding integrin on these cells. Over the first hour, hepatocyte attachment to the α1(I)CB3 and α1(I)CB8 fragments was similar (~30% in each case: Figure 4A), suggesting that the binding sites in the two fragments are roughly equivalent in their ability to support hepatocyte attachment mediated by $\alpha_1\beta_1$. Cardiac fibroblasts, with both $\alpha_1\beta_1$ and $\alpha_2\beta_1$, also attach to collagen type I and to both fragments (Figure 4B); again, the two fragments allow equivalent attachment over the first hour (~25%). As with hepatocytes, the monoclonal antibody, 3A3, abolished fibroblast attachment to α1(I)CB8 (Figure 5B). However, the $\alpha_1\beta_1$ specific antibody only reduced, but did not abolish, fibroblast attachment to collagen or α1(I)CB3. In the presence of 3A3, $\alpha_2\beta_1$ on fibroblasts was evidently able to mediate significant attachment to a site in collagen and in its α1(I)CB3 fragment. The complete inhibition by 3A3 of attachment to α1(I)CB8 strongly indicates that integrin $\alpha_2\beta_1$ does not recognize a site in α1(I)CB8. It should be noted that whereas 3A3 at 2 μg/ml did not significantly reduce hepatocyte attachment to collagen (Figure 5A),

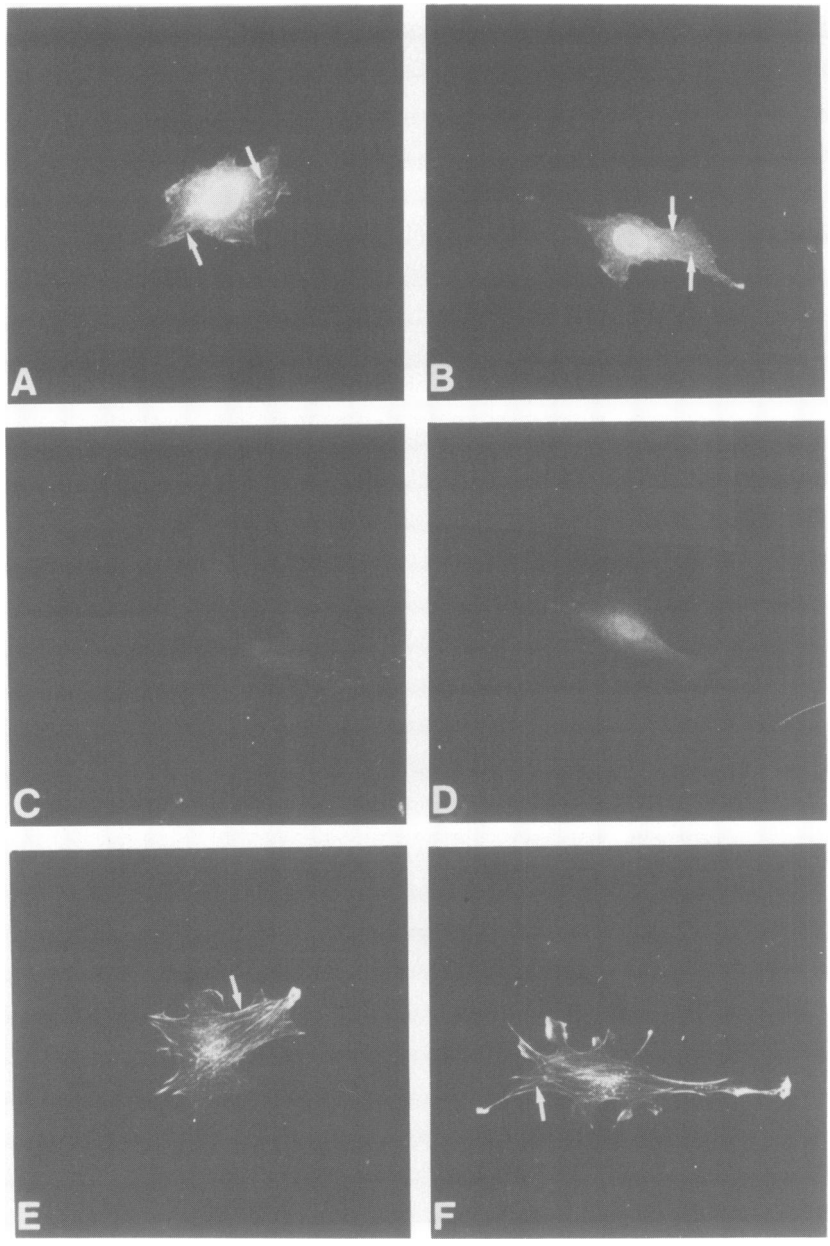

Fig. 7. Distribution of F-actin, the β_1 and $\alpha_1\beta_1$ integrin chains on Rat-1 cells cultured on collagenous substrata. Cells were seeded on coverslips coated with collagen type I (**A**, **C** and **E**) or collagen fragment α1(I)CB3 (**B**, **D** and **F**) and cultured for 4 h in serum-free media as described in Materials and methods. Cells were stained by indirect immunofluorescence for the β_1 integrin chain (A and B); for the $\alpha_1\beta_1$ integrin (C and D); or by phalloidin staining for F-actin (E and F). Magnification ×245.

the same concentration of antibody did reduce fibroblast attachment to collagen (Figure 5B). Interpretation of this finding is difficult without knowing cell surface concentrations of integrins in each cell type (see below), but it does at least suggest that $\alpha_1\beta_1$, rather than $\alpha_2\beta_1$, normally dominates the adhesive behavior of cardiac fibroblasts and that $\alpha_2\beta_1$ may therefore be less effective than $\alpha_1\beta_1$ in promoting cell attachment to collagen.

Rat-1 cells, which have only the $\alpha_2\beta_1$ collagen receptor, attached relatively poorly to collagen (Figure 4C). Their attachment to α1(I)CB3 was better than to intact collagen (Figure 4C), and comparable with that attained by hepatocytes or fibroblasts on this fragment (Figure 4A and B). Attachment of Rat-1 cells to α1(I)CB8 was very low (Figure 4C), consistent with the fibroblast results that indicated an inability of $\alpha_2\beta_1$ to bind to this fragment. Rat-1 cell attachment was not inhibited by 3A3, as expected from the low expression of $\alpha_1\beta_1$ in these cells.

The sites in α1(I)CB3 recognized by $\alpha_1\beta_1$ and $\alpha_2\beta_1$ are likely to be distinct. Otherwise, one must propose that either $\alpha_1\beta_1$ or $\alpha_2\beta_1$ can bind to a common site in α1(I)CB3 effectively enough to promote cell attachment, yet that a second site in α1(I)CB8 can be effectively recognized by $\alpha_1\beta_1$. Until information is available on the detailed structures to which $\alpha_1\beta_1$ binds, however, the issue cannot be settled.

After initial attachment, many cells form focal adhesions and organize stress fibers (Burridge *et al.*, 1988). We have previously demonstrated that rat $\alpha_1\beta_1$ appears in focal adhesions in cardiac fibroblasts cultured on collagen type

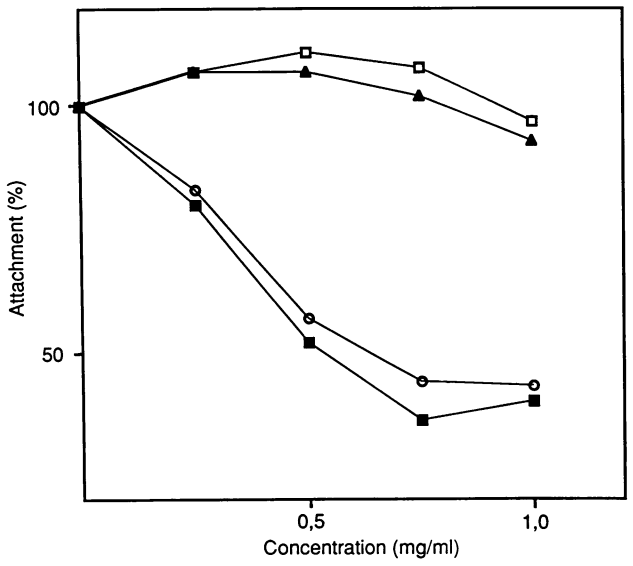

Fig. 8. Effect of GRGDTP on hepatocyte attachment to fibronectin and collagenous substrata. Hepatocytes were allowed to attach to native collagen coated at 4°C (▲--▲); denatured collagen coated at 37°C (○--○), α1(I)CB8 coated at 4°C (□--□) and to fibronectin (■--■) in the presence of the indicated concentrations of GRGDTP. Attachment was carried out at 37°C for 30 min and 1 × 10^5 cells were added to each dish. Dishes were coated as described in Materials and methods with 5 μg/ml of the various proteins. 100% attachment corresponds to ~35 000 cells for attachment to fibronectin, native collagen and α1(I)CB8, and to 10 000 cells for the attachment to denatured collagen.

I but not on fibronectin (Gullberg *et al.*, 1990). The findings that cardiac fibroblasts could form focal adhesions and organize stress fibers on α1(I)CB3 and that Rat-1 cells could do so on α1(I)CB3 (Figures 6 and 7) demonstrate that both $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins can, independently, form focal contacts and organize stress fibers.

Compared with cardiac fibroblasts, Rat-1 cells adhered less effectively to collagenous substrata (Figure 4) and formed a smaller number of β_1-integrin-containing focal adhesions (Figures 6 and 7) than the other cell types studied. This may reflect the low expression of $\alpha_1\beta_1$ in these cells, if, as suggested above, $\alpha_2\beta_1$ may be less effective than $\alpha_1\beta_1$, on a molar basis, in promoting adhesion. The poor reactivity of the α_2 specific antibody with the rat cells used in this study prevented a quantitative comparison of $\alpha_1\beta_1$ and $\alpha_2\beta_1$ in Rat-1 cells. Alternatively it is possible that Rat-1 cells express a functional variant of $\alpha_2\beta_1$. We did notice that the α_2 chain isolated from Rat-1 cells displayed a reduced M_r compared with the α_2 chain isolated from cardiac fibroblasts. This may reflect modified glycosylation of the α_2 chain in Rat-1 cells. It has been inferred from several studies that the degree of glycosylation may affect the ligand binding of integrins. A melanoma mutant showing reduced adhesion to the ECM displayed a reduced glycosylation pattern of its β_1 integrins (Öz *et al.*, 1989). In another study, the high-affinity fibronectin receptor, $\alpha_5\beta_1$, was found to be non-functional in the absence of complete oligosaccharide processing (Akiyama *et al.*, 1989). Whether the relatively poor adhesion of Rat-1 cells to collagenous substrata can be attributed to relatively low levels of $\alpha_1\beta_1$, to inherent functional differences between $\alpha_1\beta_1$ and $\alpha_2\beta_1$, or to imperfections in glycosylation of $\alpha_2\beta_1$, or indeed to some combination of these, remains to be elucidated.

We previously reported that hepatocytes adhere to and spread on denatured collagen type I only after significant lag periods (Rubin *et al.*, 1981a,b). This finding can now be interpreted as pointing to the existence of conformation dependent collagen binding sites for $\alpha_1\beta_1$. Also PC12 cell attachment to native collagen type I is completely inhibitable by 3A3, indicating that such attachment is mediated exclusively by $\alpha_1\beta_1$ (Turner *et al.*, 1989; Tomaselli *et al.*, 1990), yet PC12 cells adhere very poorly to denatured collagen type I. These data, too, strongly suggest that the binding site(s) for $\alpha_1\beta_1$ in collagen type I is conformation dependent. The CNBr peptides used in this study could renature to the triple-helical conformation as revealed by chromatic dichroism measurements (K.Åhlén and K.Rubin, unpublished observation). It is thus possible that $\alpha_1\beta_1$-mediated attachment of hepatocytes to CNBr peptides occurs to binding site(s) in the triple-helical conformation.

In an initial attempt to characterize the molecular basis for the interaction of hepatocytes with heat-denatured collagen coated at 37°C, we performed attachment experiments in the presence of the synthetic hexapeptide GRGDTP. As expected, this peptide inhibited fibroblast attachment to fibronectin (see Introduction), but, unexpectedly, it also inhibited hepatocyte attachment to denatured collagen (Figure 8). Hepatocyte attachment to native collagen type I and to α1(I)CB8 was unaffected by the GRGDTP peptide (Figure 8). These findings, taken together with the observation that 3A3 IgG completely inhibited hepatocyte attachment to native collagen (Figure 5), suggests that the RGD sequence in collagen type I is non-functional when the collagen molecule is in the triple-helical conformation but functional when the collagen is in random coil conformation. This is in agreement with what has been shown for collagen type VI (Aumailley *et al.*, 1989) and collagen type IV (Vandenberg *et al.*, 1991). Since hepatocytes seem to lack vitronectin receptors (Gullberg *et al.*, 1989) the RGD dependent attachment to denatured collagen coated at 37°C is most likely mediated by $\alpha_5\beta_1$ present on the hepatocytes (Johansson *et al.*, 1987).

In conclusion, our data show: (i) that $\alpha_3\beta_1$ is not involved in the initial attachment of hepatocytes, cardiac fibroblasts or Rat-1 cells to collagen type I; (ii) that binding sites for integrin $\alpha_1\beta_1$ are present in two non-overlapping triple-helical fragments derived from collagen type I; (iii) that integrin $\alpha_2\beta_1$ binds to only one of these fragments; and (iv) that hepatocyte attachment to heat-denatured collagen type I, unlike that of the native molecule, is RGD dependent. We interpret these findings as suggesting that $\alpha_1\beta_1$ and $\alpha_2\beta_1$ recognize distinct sites and that the sites recognized by $\alpha_1\beta_1$ are conformation dependent.

Materials and methods

Reagents

Calf collagen type I was isolated from calf skin by pepsin cleavage in acetic acid (Miller and Rhodes, 1982) or obtained commercially (Vitrogen 100, Collagen Corporation, Palo Alto, CA, USA), GRGDTP and GRGESP peptides were from Telios Pharmaceuticals (San Diego, CA, USA). CNBr-activated Sepharose 4B, protein A–Sepharose 4B and S-Sepharose Fast Flow were from Pharmacia AB (Uppsala, Sweden). Lactoperoxidase, goat anti-mouse IgG coupled to agarose, glucose oxidase type II and pepstatin A were from Sigma Chemical Co. (St Louis, MO, USA). Leupeptin was from Boehringer Mannheim (Mannheim, Germany) and aprotinin was from

Bayer (Leverkusen, Germany). Texas Red avidin D, biotinylated horse anti-mouse IgG and biotinylated goat anti-rabbit IgG were from Vector (Burlingame, CA, USA). Rhodamine-labeled phalloidin was from Molecular Probes (Eugene, OR, USA).

Collagen fragments

Calf skin collagen type I (500 mg) was dissolved in 100 ml of 70% (v/v) formic acid containing CNBr (12 mg/ml) and digested at room temperature overnight. Digestion was terminated by dilution with water and freeze drying. The cleaved product was then subjected to cation-exchange chromatography on 20 ml of S-Sepharose Fast Flow at room temperature (Bateman *et al.*, 1986). Eluted fractions were subjected to 10–15% SDS–PAGE and stained with Coomassie blue. Material eluting at the position expected of α1(I)CB3 and with an apparent molecular weight in SDS–PAGE corresponding to a pure sample of α1(I)CB3 kindly provided by Dr Rupert Timpl (Munich, Germany), was pooled and freeze dried. Human placenta α1(I)CB8 was prepared as described (Fitzsimmons *et al.*, 1986). The purified cyanogen bromide fragments were free of contaminants as judged by silver staining of SDS–PAGE gels and stored at 4°C in 0.1 M acetic acid.

Cells

Hepatocytes were obtained by a collagenase perfusion method as described by Öbrink (1982). Monolayer cultures of primary neonatal rat cardiac fibroblasts were established by differential attachment of cells obtained after collagenase digestion of hearts as previously described (Borg *et al.*, 1984). Primary rat cardiac fibroblasts were grown in DMEM medium supplemented with 10% fetal bovine serum and used between passages 5 and 15. The fibroblastic cell line, Rat-1 clone N1, was kindly donated by Dr Bengt Westermark, Department of Pathology, Uppsala University.

Antibodies

Anti-β_1 integrin IgG was raised as described (Gullberg *et al.*, 1989). The isolation and characterization of the monoclonal antibody, 3A3, which specifically recognizes the rat collagen/laminin $\alpha_1\beta_1$ receptor and which perturbs PC12 cell adhesion to collagen and laminin, has been described elsewhere (Turner *et al.*, 1989; Tawil *et al.*, 1990). The α_2 integrin specific antibody was raised against human platelet α_2 and kindly provided by Dr Klaus von der Mark (Abt. Rheumatologie, Max-Planck Institute, Erlanger, Germany). Two α_3 specific antibodies were used in the present study. The first, denoted anti-α_3(pep), was made against a synthetic peptide corresponding to the C-terminal 14 residues of the chicken α_3 integrin subunit as described (Hynes *et al.*, 1989) and was a gift from Dr Richard Hynes, Center for Cancer Research, MIT, Cambridge, MA. The second, denoted anti-rat α_3, was made against rat $\alpha_3\beta_1$, affinity purified and then absorbed on $\alpha_5\beta_1$–Sepharose to remove all anti-β_1 antibodies as described (Tomaselli *et al.*, 1990).

Polyacrylamide gel electrophoresis and immunoblotting

Electrophoresis and immunoblot analysis was performed as described (Blobel and Doberstein, 1975; Gerton *et al.*, 1982). Molecular weight standards were from Bio-Rad Laboratories. Reduced samples were treated with 10 mM DTT followed by alkylation with 50 mM iodoacetamide. The molecular weights of the blotted proteins were estimated by relating their position to ^{125}I-labeled marked proteins (Sigma MW-SDS-200 marker protein kit supplemented with fibronectin) transferred from the SDS–polyacrylamide gel.

Immunoprecipitation and affinity chromatography

Confluent fibroblasts and Rat-1 cells, or overnight cultures of hepatocytes, were labeled with ^{125}I in culture dishes as described. Immunoprecipitation of extracts of ^{125}I surface-labeled cells was performed as described (Gullberg *et al.*, 1990). Sequential affinity chromatography of detergent-solubilized hepatocyte proteins was performed on lentil lectin– and collagen type I–Sepharose (Gullberg *et al.*, 1990). EDTA-eluted proteins were separated on SDS–PAGE and visualized by silver staining. ^{125}I surface-labeled rat fibroblasts and Rat-1 cells were solubilized and cell extracts were subjected to affinity chromatography on collagen I–Sepharose as described earlier (Gullberg *et al.*, 1990).

Cell attachment assay

Cell attachment and antibody inhibition assays were performed in 24-well culture plates (Nunc, Roskilde, Denmark) essentially as described by Gullberg *et al.* (1989). Individual ECM components or CB peptides were conjugated by glutaraldehyde to BSA-coated on plastic dishes (Gullberg *et al.*, 1989). The coating procedure was carried out at 4°C unless otherwise stated; CB peptides were not denatured prior to coating. In separate experiments, using CNBr fragments labeled with ^{125}I by the Bolton–Hunter method (Bolton and Hunter, 1973), no difference in coating efficiency between α1(I)CB8 and α1(I)CB3 was noted. Following coating, the plates to be used for the primary rat cardiac fibroblasts and the Rat-1 cells were incubated with 1.5% BSA in PBS at 37°C for 2 h. In the case of primary rat cardiac fibroblasts and Rat-1 cells, 1×10^5 cells were added per well, whereas for hepatocytes 2×10^5 cells per well were added unless otherwise stated. Antibody inhibition assays were performed as previously described (Gullberg *et al.*, 1989).

Radioreceptor binding assay

Laminin binding integrins were isolated from Rat-1 cells and cardiac fibroblasts by affinity chromatography on human laminin–Sepharose as described (Gehlsen *et al.*, 1988, 1989). The composition of the isolated proteins was checked by SDS–PAGE, immunoblotting and immunoprecipitation; only $\alpha_3\beta_1$ could be identified in the eluates from laminin–Sepharose. The radioreceptor assays were performed in microtiter plates coated with 1–2 μg/ml of mouse EHS tumor laminin, human plasma fibronectin or 5 μg/ml of bovine collagen type I as described (Gehlsen *et al.*, 1989).

Immunofluorescence

Primary fibroblasts and Rat-1 cells were cultured on collagen type I-, CB-peptide or fibronectin-coated poly-L-lysine-treated coverslips for 4 h. Coverslips were rinsed in PBS and cells were fixed with 2% paraformaldehyde for 15 min, washed in PBS and permeabilized with 0.5% Triton X-100 in PBS for 30 min and incubated with 0.1 M glycine, pH 7.4 for 1 h followed by 0.5% BSA in PBS (PBS–BSA) for 30 min or overnight. The monoclonal 3A3 antibody (5–10 μg/ml), anti-β_1 integrin IgG (40 μg/ml) and control IgG, were incubated with fixed and permeabilized fibroblasts at 37°C for 45 min. The coverslips were rinsed with PBS–BSA and incubated with biotinylated anti-mouse or anti-rabbit IgG at 37°C for 30 min, rinsed with PBS–BSA, incubated with Texas Red-labeled avidin at 37°C for 30 min and rinsed in PBS–BSA. Following a final rinse in distilled water the coverslips were mounted in Fluoromount-G. Actin staining was performed by incubating fixed and permeabilized cells with rhodamine-labeled phalloidin for 30 min, washed and mounted.

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