Microfibril-associated glycoprotein-2 (MAGP)-1 and MAGP-2 are small structurally related glycoproteins that are specifically associated with fibrillin-containing microfibrils. MAGP-2, unlike MAGP-1, contains an RGD motif with potential for integrin binding. To determine if the RGD sequence is active, a series of cell binding assays was performed. MAGP-2 was shown to promote the attachment and spreading of bovine nuchal ligament fibroblasts when coated onto plastic wells in molar quantities similar to those of fibronectin. In contrast, ~10-fold more MAGP-1 was required to support comparable levels of cell adhesion. The fibroblast binding to MAGP-2 was completely inhibited if the peptide GRGDSP or the MAGP-2-specific peptide GVSGQRGDDVTITSET was added to the reaction medium at a 10 μM final concentration. The control peptide GRGESP had no effect on the interaction. These findings indicate that the cell interaction with MAGP-2 is an RGD-mediated event. A monoclonal antibody to human αvβ3 integrin (LM609) almost completely blocked cell attachment to MAGP-2 when added to the medium at 0.5 μg/ml, whereas two monoclonal antibodies specific for the human β1 integrin subunit, 4B4 (blocking) and QE2.E5 (activating), had no effect even at 10 μg/ml. Fetal bovine aortic smooth muscle cells, ear cartilage chondrocytes, and arterial endothelial cells and human skin fibroblasts and osteoblasts were also observed to adhere strongly to MAGP-2. In addition, each cell type was able to spread on MAGP-2 substrate, with the exception of the endothelial cells, which remained spherical after 2 h of incubation. The binding of each cell type was blocked when the anti-αvβ3 integrin antibody was included in the assay, indicating that αvβ3 integrin is the major receptor for MAGP-2 on several cell types. Thus, MAGP-2 may mediate interactions between fibrillin-containing microfibrils and cell surfaces during the development of a variety of tissues.

MAGPs are a two-member family of small structurally related glycoproteins, MAGP-1 (31 kDa) and MAGP-2 (25 kDa), which are specifically associated with fibrillin-containing microfibrils (1,3). Fibrillin-containing microfibrils (10–12-nm diameter) are important structural components of the extracellular matrix of most connective tissues. In elastic tissues such as arteries, lung, and elastic ligaments, these microfibrils are components of elastic fibers, in association with the elastic protein, elastin. Fibrillin-containing microfibrils can also occur as elastin-free bundles in tissues such as ocular zonule, skeletal muscle, and kidney glomerulus (2–4). The major structural components of these microfibrils are rod-like 350-kDa glycoproteins named fibrillin-1 and -2, which appear to be arranged as parallel bundles of 4–8 molecules joined in series in a head-to-tail manner (5–8). Fibrillin-1 and -2 have distinct, but overlapping, spatiotemporal tissue distributions, indicating that the microfibrils have structural and functional heterogeneity (9–10). It is unclear if fibrillin-1 and -2 form separate populations of microfibrils or if they can coexist in the same microfibril. Mutations in the genes for fibrillin-1 and -2 have been linked to the heritable connective tissue disorders Marfan syndrome and congenital contractual arachnodactyly, respectively (11,12).

Despite being the subjects of intensive investigation, the precise molecular composition, architecture, and structural and functional heterogeneity of the microfibrils are still being elucidated (3,5). An increasing number of proteins have been identified in association with fibrillin-containing microfibrils. In addition to MAGP-1 and MAGP-2, these include microfibril-associated proteins 1, 3, and 4 (13–15); latent transforming growth factor-β1-binding proteins (16–18); fibulins (19); and emilin (20). In most instances, it is unclear if the protein forms part of the microfibril or is adhered to its surface.

There is strong biochemical and immunoelectron microscopic evidence that MAGP-1 and MAGP-2 are covalently linked by disulfide bonding to fibrillin-containing microfibrils within tissues (21). MAGP-1 co-distributes with most, if not all, fibrillin-1-containing microfibrils and is localized in a specific periodic manner on the beads of the “beads-on-a-string” structure of these microfibrils revealed by the rotary shadowing technique (4,22,23). This suggests that MAGP-1 may be an integral component of microfibrils of this type. MAGP-2 exhibits more restricted tissue and developmental patterns of distribution, suggesting that MAGP-2 has a more specialized role in microfibril biology (22). Cloning of MAGP-1 and MAGP-2 revealed that they each contain a characteristic central motif with close sequence similarity between the molecules, including precise alignment of seven cysteine residues (1, 24). It is considered likely that this cysteine-rich region is involved in the interactions of MAGPs with other components of the microfibril. In contrast, the other regions of MAGP-1 and MAGP-2 were found to be very divergent in structure. Evidence indicates that the N-terminal region of MAGP-1 contains binding sequences for tropoelastin and type VI collagen, and it is possible that the glycoprotein functions on the surface of the microfibrils, as an elastin-binding protein during elastinogenesis and as an anchoring protein mediating the interaction of fibrillin-containing microfibrils and type VI collagen microfibrils (25–27). In con-
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The N-terminal region of MAGP-2 was found to contain an RGD motif (1), suggesting that the glycoprotein may have integrin binding activity (28, 29). Using an in vitro cell binding assay, we have now shown that MAGP-2 interacts with a wide range of cell types in an RGD-dependent manner via \( \alpha_v\beta_3 \) integrin.

**EXPERIMENTAL PROCEDURES**

**Materials**—MAGP-1 and MAGP-2 were prepared from fetal bovine nuchal ligament as described previously (21). The RGD-containing synthetic peptide MP25A (GVSGQRGGVTTVTTSET, corresponding to amino acids 25–41 of the deduced primary structure for bovine MAGP-2) was prepared by Chiron Mimotopes (Melbourne, Australia). Other peptides and bovine fibronectin were purchased from Life Technologies, Inc. Anti-Mimotopes (Melbourne, Australia). Other peptides and bovine fibronectin were purchased from Life Technologies, Inc. Anti-human \( \alpha_v\beta_3 \) integrin monoclonal antibody LM609 (30) was purchased from Chemicon International, Inc. (Temecula, CA). Anti-human \( \beta_1 \) integrin monoclonal antibodies 4B4 (31) and QE2.E5 (32) were obtained from Beckman Coulter Inc. and Dr. R. J. Faull (University of Adelaide), respectively. Anti-\( \beta_1 \) integrin antibody P5D2 (33) was a kind gift from Dr. E. A. Wayner (Fred Hutchinson Cancer Research Center, Seattle, WA).

Nuchal ligament fibroblasts, ear cartilage chondrocytes, and aortic smooth muscle cells were grown from tissues of 210-day-old bovine fetuses using the explant technique. Endothelial cells were obtained from the umbilical arteries of 210-day-old bovine fetuses using the method of Wall et al. (34). Human skin fibroblasts obtained from normal adult subjects have been described previously (35). Human osteoblastic cells, grown from trabecular bone explants of normal adults, were a kind gift from Dr. D. Haynes (Department of Pathology, University of Adelaide). All of the above cell types were passaged less than four times before use in the binding experiments. Human mononuclear cells were freshly prepared from peripheral blood using a standard Ficoll density gradient method. Human cell lines that were used in the binding assay included MO7e (megakaryoblastic) (36), NALM-6 (null lymphoblastoid) (37), BALM-1 (B lymphocytic) (38), and T47D (breast carcinoma) (American Type Culture Collection).

**Cell Adhesion Assay**—The cell adhesion assay was based on that of Sakamoto et al. (39). Cell culture-grade micrometer plates were coated with MAGP-1, MAGP-2, or fibronectin (0–200 ng/well) for 18 h at 4 °C, followed by blocking with BSA (1 mg/ml) in phosphate-buffered saline for 2 h and rinsing three times with phosphate-buffered saline for 5 min. For cells that adhere to plastic, confluent cells were released by treatment with trypsin (0.1%) in Dulbecco’s phosphate-buffered saline containing 0.5 mM EDTA for 5 min at 37 °C. The suspended cells were washed once with Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and twice with binding buffer (Hepes-buffered Dulbecco’s modified Eagle’s medium containing 1 mg/ml BSA) before being added to the wells at a density of 2.5 \( \times 10^4 \) cells/well in 100 \( \mu \)l of binding buffer. Cells that form suspensions in culture were suspended directly in binding buffer. The adhesion assay was conducted at 37 °C for 2 h, and in some instances, cells were photographed under phase-contrast using a Nikon Diaphot inverted microscope. Non-adhering cells were removed by gentle washing with phosphate-buffered saline. Cell binding activity was measured by assay for hexosaminidase with \( p \)-nitrophenyl N-acetyl-\( \beta \)-D-glucosaminide substrate at \( A_{405\text{nM}} \) (40).

In some experiments, a synthetic peptide (0.01–50 \( \mu \)M) was included in the binding reaction. For experiments involving monoclonal antibodies, the cells were preincubated for 30 min at room temperature with the antibody (0.02–10 \( \mu \)g/ml), followed by incubation in the wells at 37 °C for 2 h.

**RESULTS**

MAGP-2 Promotes the Adherence and Spreading of a Range of Cell Types from Fetal Bovine Tissues—Using a solid-phase cell attachment assay, MAGP-2 was shown to promote the adherence of nuchal ligament fibroblasts when microrretter wells were coated with subpicomole quantities of the protein (Fig. 1A). These amounts were similar on a molar basis to those required for fibronectin, which contains an active RGD integrin-binding motif, to promote binding of the cells. In contrast, 10-fold greater molar quantities of structurally related MAGP-1, which lacks an RGD motif, were required to promote equivalent cellular adherence. The adherence and spreading of cells was monitored by light microscopy before the removal of unbound cells prior to the assay (Fig. 1B). Cells in control wells coated with BSA only (panel a), MAGP-2 (0.25 pmol/well) (panel b), or MAGP-1 (0.5 pmol/well) (panel c). Note the extensive spreading of cells on MAGP-2 substrate. Bar = 50 \( \mu \)m.

In some experiments, a synthetic peptide (0.01–50 \( \mu \)M) was included in the binding reaction. For experiments involving monoclonal antibodies, the cells were preincubated for 30 min at room temperature with the antibody (0.02–10 \( \mu \)g/ml), followed by incubation in the wells at 37 °C for 2 h.
higher quantities of MAGP-2 were required to support a level of endothelial cell binding equivalent to that of other cell types, suggesting that endothelial cell attachment is of lower affinity. This is supported by the morphological observation that, after the 2-h incubation, the endothelial cells were adhering, but not spreading, on MAGP-2 substrate. In contrast, the other cell types showed extensive spreading within this time period (data not shown). The adherence of each cell type to MAGP-2 was completely blocked by 5 mM EDTA, indicating that the interaction was a calcium-dependent process (data not shown).

**MAGP-2 Binds to Cells via Its RGD Sequence**—To determine if the cell adhesion to MAGP-2 was mediated through its RGD sequence, the binding assay was conducted in the presence of (i) a short peptide (GRGDSP) that is known to inhibit RGD-dependent binding to a range of integrins (29), (ii) a 17-amino acid peptide corresponding to the region of MAGP-2 containing the RGD motif, or (iii) a control peptide (GRGESP) that has no integrin binding properties (Fig. 3A). Both RGD-containing peptides were shown to inhibit significantly the binding of nuchal ligament fibroblasts to MAGP-2 at a peptide concentration of 2.5 μM and to inhibit the interaction completely at a concentration of 50 μM. In contrast, the control peptide showed no inhibition of the interaction. These findings were confirmed morphologically as shown in Fig. 3B. In the absence of peptide (Fig. 3B, panel a) or in the presence of the control peptide (panel b), the fibroblasts adhered and spread extensively on MAGP-2 substrate. However, in the presence of either RGD-containing peptide (10 μM), the cells failed to adhere and retained a spherical appearance (Fig. 3B, panels c and d). Since these RGD-containing peptides specifically inhibited cellular adherence to MAGP-2, it is evident that its RGD motif was mediating the interaction, most likely via a cell-surface receptor(s) of the integrin family.

**Specific Inhibition of Cell Adhesion to MAGP-2 by Anti-αβ₂ Integrin Antibody LM609**—A panel of antibodies known to modulate the binding activity of human β₂ and αβ₂ integrins was tested in the cell binding assay with MAGP-2. Preliminary immunofluorescence studies had shown that each antibody strongly stained bovine nuchal ligament fibroblasts, with the exception of anti-β₂ integrin antibody P5D2, which did not stain the cells, suggesting that it did not recognize the bovine form of the integrin (data not shown). Therefore, antibody 4B4 was used as the blocking anti-β₂ integrin antibody in the cell binding studies. Experiments showed that the binding of nuchal ligament fibroblasts to MAGP-2 could be almost completely inhibited by incubation of the cells with blocking anti-αβ₂ integrin antibody LM609 (at concentrations above 1 μg/ml) during the binding assay (Fig. 4A). In contrast, antibodies 4B4 and QE2.E5 (blocking and activating antibodies, respectively, recognizing the human β₂ integrin subunit) had no effect in the binding assay even at a concentration of 10 μg/ml. Consistent with the above findings, microscopic examination showed that the fibroblasts treated with the anti-αβ₂ integrin antibodies (0.5 μg/ml) did not adhere and had a spherical morphology after incubation on MAGP-2 substrate (Fig. 4B, panel c). This appearance was indistinguishable from that of control cells incubated in wells lacking MAGP-2 (Fig. 4B, panel a). In contrast, neither of the anti-β₁ integrin antibodies 4B4 and QE2.E5 (10 μg/ml) affected the adherence and spreading of the cells on MAGP-2 substrate (Fig. 4B, panels b and d). In a separate experiment, both of these antibodies were shown to modulate the binding of nuchal ligament fibroblasts to fibronectin (Fig. 5). This confirmed that both antibodies were functionally active against the bovine β₁ integrin subunit and that this receptor is expressed on the surface of the fibroblasts. Overall, the findings indicate that nuchal ligament fibroblasts interact with MAGP-2 via αβ₂ integrin and that MAGP-2 is not recognized by the β₁ family of integrins. Anti-αβ₂ integrin antibody LM609 also strongly inhibited the bind-
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A bovine fibroblasts from fetal nuchal ligament (2.5 $\times$ 10^4/well) were treated with antibody at concentrations of 0.002–10 $\mu$g/ml as substrate. $\square$, anti- $\alpha_\beta_3$ integrin antibody LM609; $\bullet$, anti- $\beta_3$ integrin antibody 4B4 (blocking); $\triangle$, anti- $\beta_1$ integrin antibody QE2.E5 (activating). Cell adhesion is expressed as a percentage of the $A_{405}$ nm value obtained in the absence of antibody treatment. Results are the means $\pm$ S.D. of quadruplicate determinations. B, shown is cell morphology prior to removal of non-adhering cells. Incubations were conducted with no MAGP-2 or antibody (control) (panel a), no antibody (panel b), anti- $\alpha_\beta_3$ integrin antibody LM609 (0.5 $\mu$g/ml) (panel c), and anti- $\beta_3$ integrin antibody 4B4 (10 $\mu$g/ml) (panel d). Note the lack of adherence and spreading in the presence of the antibody to $\alpha_\beta_3$ integrin. Bar $= 50$ $\mu$m.

To determine the species specificity of cell adhesion to MAGP-2, the cell binding assay was repeated using a variety of human cell types. Cell binding assays showed that skin fibroblasts and osteoblasts from adult human sources also adhered to MAGP-2. The attachment of both cell types to MAGP-2 substrate was almost completely inhibited by anti-$\alpha_\beta_3$ integrin antibody LM609, confirming that this integrin was also the major mediator of the interaction with human cells (Fig. 6). A blocking anti-human $\beta_3$ integrin antibody (P5D2) had no effect on the cell binding to MAGP-2. Overall, the results indicate that MAGP-2 specifically interacts with a range of bovine and human cell types via $\alpha_\beta_3$ integrin.

A number of human cell types were found not to adhere to MAGP-2. These included peripheral blood monocytes and several cancer cell lines, including MO7e (megakaryoblastic), NALM-6 (null lymphoblastoid), BALM-1 (B lymphocytic), and T47D (breast carcinoma). Interestingly, the BALM-1 cells stained very strongly by immunofluorescence with anti-$\alpha_\beta_3$ integrin antibody LM609, whereas the other cell lines stained relatively weakly (data not shown). The failure of the BALM-1 cells to adhere to MAGP-2 may be attributed to the integrin being present in an inactive low affinity conformation on these cells. Modulation of integrin affinity has been extensively documented (28), and several cell lines of hematopoietic origin have

**Table I**

| Cell type                  | Cell adhesion $^a$ (%) |
|----------------------------|------------------------|
| Nuchal ligament fibroblast | 35.3 $\pm$ 1.1         |
| Ear cartilage chondrocyte  | 36.3 $\pm$ 7.5         |
| Aortic smooth muscle cell  | 40.2 $\pm$ 2.5         |
| Arterial endothelial cell  | 38.4 $\pm$ 3.7         |

$^a$ Cell adhesion is expressed as a percentage of the $A_{405}$ nm value obtained in the absence of antibody treatment. Results are the means $\pm$ S.D. of quadruplicate determinations.
been shown to express integrins on their surfaces in low affinity conformations (41, 42).

**DISCUSSION**

Previous sequence analysis of human and bovine MAGP-2 showed a conserved RGD-containing sequence (QRGDDVT) to be present in both proteins (1). Surface probability analysis indicated that the sequence was likely to reside on the surface of the MAGP-2 molecule. These observations suggested that the RGD sequence in MAGP-2 might have integrin binding properties. In this study, we have established that a variety of human and bovine cell types do specifically adhere and spread on subpicomole quantities of bovine MAGP-2. Cells that were found to interact with MAGP-2 include fetal bovine nuchal ligament fibroblasts, aortic smooth muscle cells, ear cartilage chondrocytes, and arterial endothelial cells and adult human skin fibroblasts and osteoblasts. The binding could be almost totally inhibited by RGD-containing peptides and by a blocking anti-human αβ3 integrin monoclonal antibody. Blocking anti-β3 integrin antibodies showed no effect on cell binding to MAGP-2. Overall, the results indicate that αβ3 integrin is the major receptor for MAGP-2 on all of the cell types shown to adhere to the protein.

Integrin αβ3 is the most promiscuous of the RGD-dependent integrins (29, 43). It has been shown to mediate the adhesion and spreading of many cell types on a wide range of matrix macromolecules, including vitronectin, fibronectin, tenascins, thrombospondin, laminin-1, and osteopontin (44) and, of particular interest, the microfibrillar proteins fibrillin-1 and -2 (39, 45, 46). Upon ligand binding, αβ3 integrin forms part of focal adhesion complexes, where it can mediate the interaction of the above matrix components with the actin-containing cytoskeleton, important for cell spreading and migration (43). In addition, there is evidence that the integrin is involved in several other cell signaling pathways (47). Integrin αβ3 has previously been identified on the surface of each of the cell types found to adhere to MAGP-2 in this study (39, 48–50). There is some debate about the presence of significant levels of active αβ3 integrin on osteoblastic cells. Saito et al. (51) detected only very low levels of this integrin on cultured human osteoblasts. Gronthos et al. (49) presented strong evidence that the integrin is present on cultured osteoblastic cells, but that it might be in a non-active form. However, more recently, Wendel et al. (52) showed that the integrin is active on bovine osteoblasts, where it mediates the interaction of the cells with the novel keratin sulfate proteoglycan osteoadherin. Therefore, the observation that MAGP-2 interacts with human osteoblasts via αβ3 integrin is consistent with the above study. It is interesting that, in contrast to other adhering cell types, the arterial endothelial cells adhered to, but did not spread, on MAGP-2 substrate. This observation indicates that the cell behavioral consequences of the αβ3 integrin-mediated interaction with MAGP-2 are not identical for all adhering cell types. This finding is reminiscent of the study of Joshi et al. (53), who reported that endothelial cells adhere to tenasin in an RGD-dependent manner, but do not spread on this substrate. The authors suggested that the tenasin may be eliciting or enhancing a signaling function on the endothelial cells. Thus, it is also possible that the interaction of MAGP-2 and αβ3 integrin on such cells serves predominantly as a signaling mechanism rather than facilitating the anchoring and spreading function observed with other cell types.

In previous immunolocalization studies, MAGP-2 has been identified with fibrillin-containing microfibrils in fetal tissues such as nuchal ligament, aortic intima, skin, skeletal muscle, and kidney mesangium (22). These MAGP-2-staining microfibrils were often observed closely adjacent to the surfaces of cells. Therefore, it is likely that MAGP-2 can interact in vivo with several of the cell types examined in this study. It should be noted, however, that MAGP-2 also bound to fetal aortic smooth muscle cells and chondrocytes even though the protein was not identified in the corresponding tissues (22). In developing nuchal ligament, the period of maximal MAGP-2 expression corresponds to the period of highest fibrillin-1 expression that, in turn, correlates with the early stages of elastinogenesis (22, 54). In this period, nascent elastic fibers consist of bundles of fibrillin-containing microfibrils coated with newly forming elastin, lying closely adjacent and parallel to the extracellular surface of cells (2). Thus, it has been proposed that the cell directs the deposition and orientation of the elastic fiber components during elastinogenesis, resulting in elastic fibers with tissue-specific morphology, e.g. thick fibers in nuchal ligament, fenestrated lamellae in aorta, and a range of structures from thin elastic fibers to elastin-free microfibrillar bundles in skin (2, 3).

To achieve tissue-specific morphology, these processes are likely to be modulated by variation in the expression and function of intracellular, cell surface-associated, and extracellular proteins. At the cell surface, the elastin receptor has been implicated in elastic fiber assembly (55, 56), and it has been suggested that αβ3 integrin may also be involved in the process through its interaction with fibrillin-1 (39). Integrins have already been found to play major roles in the cell-surface organization of fibronectin polymers (57). The evidence presented here indicates that MAGP-2 may also be involved in cell-surface interaction with fibrillin-containing microfibrils. The presence of an active RGD integrin-binding site on this relatively small microfibril-associated protein suggests that interaction with αβ3 integrin is a major function of the molecule. It is interesting that the location of this RGD motif corresponds to the putative elastin/type VI collagen-binding site on its structural relative MAGP-1, considered to be important for microfibril/matrix interactions (27). The above points, together with the restricted tissue distribution of MAGP-2, suggest that the molecule is involved in modulation of microfibril interactions with cell surfaces in particular tissue environments. Since MAGP-2 has been identified in adult kidney mesangium (22), it is evident that the protein has some enduring role in microfibril biology. Rather than being a constitutive structural component of all microfibrils, MAGP-2 may be important for the generation and maintenance of microfibrils and elastic fibers with tissue-specific morphological, physical, and cell biological properties. An intriguing possibility is that, during the above processes, the presence or absence of MAGP-2 on the surface of the microfibrils may influence the interaction of cell-surface αβ3 integrin with fibrillin-1 within the microfibrils, leading to distinct biological consequences.

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