Signal transduction by β1 integrin receptors in human chondrocytes *in vitro*: collaboration with the insulin-like growth factor-I receptor

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We have examined the mechanism by which collagen-binding integrins co-operate with insulin-like growth factor-I (IGF-I) receptors (IGF-IR) to regulate chondrocyte phenotype and differentiation. Adhesion of chondrocytes to anti-β1 integrin antibodies or collagen type II leads to phosphorylation of cytoskeletal and signalling proteins localized at focal adhesions, including α-actinin, vinculin, paxillin and focal adhesion kinase (FAK). These stimulate docking proteins such as Shc (Src-homology collagen). Moreover, exposure of collagen type II-cultured chondrocytes to IGF-I leads to co-immunoprecipitation of Shc protein with the IGF-IR and with β1, α1 and α5 integrins, but not with α3 integrin. Shc then associates with growth factor receptor-bound protein 2 (Grb2), an adaptor protein and extracellular signal-regulated kinase. The expression of the docking protein Shc occurs only when chondrocytes are bound to collagen type II or integrin antibodies and increases when IGF-I is added, suggesting a collaboration between integrins and growth factors in a common/shared biochemical signalling pathway. Furthermore, these results indicate that focal adhesion assembly may facilitate signalling via Shc, a potential common target for signal integration between integrin and growth-factor signalling regulatory pathways. Thus, the collagen-binding integrins and IGF-IR co-operate to regulate focal adhesion components and these signalling pathways have common targets (Shc–Grb2 complex) in subcellular compartments, thereby linking to the Ras–mitogen-activated protein kinase signalling pathway. These events may play a role during chondrocyte differentiation.

Key words: alginate, IGF-1 receptor, Shc, Grb2, Erk, immunoprecipitation.

INTRODUCTION

Extracellular matrix proteins, such as collagens, fibronectin, laminin and vitronectin, affect cell proliferation, differentiation and morphogenesis [1,2]. The interaction between chondrocytes and matrix proteins is mediated largely by the β1 subfamily of integrins [2–6]. The interaction between matrix proteins and integrins, leading to the specific activation of intracellular signalling proteins, has been studied in many cell types, but not yet in chondrocytes.

Integrins are heterodimeric transmembrane molecules composed of an α- and a β-subunit. They consist of a large extracellular domain, a transmembrane domain and a short cytoplasmic domain. The extracellular domain is able to bind to various extracellular matrix ligands, such as collagens, fibronectin, laminin and vitronectin [7]. In addition to their function as cell adhesion receptors, integrins play an important role as signalling receptors. Integrin-mediated adhesion to extracellular proteins can activate multiple cytoskeletal-associated proteins, such as paxillin [8], tensin [9] and intracellular signalling proteins such as FAK (focal adhesion kinase, a non-receptor protein-tyrosine kinase) [10]. It is well known that tyrosine phosphorylation of FAK is induced by cell–extracellular matrix interaction, by overexpression of the cytoplasmic domain of the integrin β subunit and by other growth factors [11,12]. One downstream signalling protein in the integrin-generated signalling pathway is the adaptor protein Shc (Src-homology collagen). Shc proteins are modular molecules characterized by a C-terminal SH2 domain and an N-terminal phosphotyrosine-binding domain [13]; it has been reported that these docking proteins are involved in signal transduction between tyrosine kinases and Ras proteins [14,15]. Shc proteins are activated by receptor tyrosine kinases, such as insulin receptor [16], fibroblast growth factor receptor [17] and epidermal growth factor receptor [13]. Shc proteins also become tyrosine phosphorylated by specific ligand–receptor interactions, which have no tyrosine kinase activity, by activating cytoplasmic tyrosine kinases [18]. In addition, tyrosine phosphorylated Shc forms a complex with the growth factor receptor-bound protein 2 (Grb2) adaptor protein [19] and the Shc–Grb2 complex induces Ras activation via Grb2-associated son of sevenless, a cytoplasmic GTP exchange protein [20,21]. Ras then stimulates the extracellular signal-regulated kinase 1 (Erk1)/Erk2 mitogen-activated protein (MAP) kinase cascade [22], which plays an important role in cellular differentiation and growth.

Several observations suggest that integrins interact with growth factors and that this may be important for cell adhesion, differentiation, growth and survival in different cell types [23–26]. Insulin-like growth factor I (IGF-I) is known to stimulate the differentiation of chondrocytes and matrix synthesis (incorporation of sulphate into proteoglycans) *in vivo* and *in vitro* [27,28]. It has been reported that most normal cells become susceptible to the action of growth factors, a process necessary for cell survival, only after integrin-mediated adhesion to the extracellular matrix [29]. Furthermore, several lines of evidence indicate the presence of different growth factor receptors in isolated focal adhesion complexes [30]. IGF-I has been shown to stimulate the expression and function of integrins in chondrocytes [31]. However, the influence of integrin and specific growth factor interactions on chondrocyte functions and differentiation is at present not fully understood.

The goal of this study was to investigate the hypothesis that

Abbreviations used: Erk, extracellular signal-regulated kinase; FAK, focal adhesion kinase; Grb2, growth factor receptor-bound protein 2; IGF-I, insulin-like growth factor I; IGF-IR, IGF-I receptor; MAP, mitogen-activated protein; p-Tyr, anti-phosphotyrosine; Shc, Src-homology collagen.

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the integrin-dependent responses modulated by exogenous IGF-I in human chondrocytes require integrin-mediated adhesion to specific cartilage matrix proteins (collagen type II). The results of this study show that collagen-binding integrins and activated IGF-I receptors (IGF-IR) co-immunoprecipitate with intracellular signalling adaptor proteins such as Shc, and this common target forms the Shc–Grb2–Erk complex leading to the Ras–MAP kinase signalling pathway. These mechanisms most likely prevent chondrocyte dedifferentiation to fibroblast-like cells and chondrocyte death.

MATERIALS AND METHODS

Materials

Polyclonal anti-integrin antibodies used for immunoprecipitation were anti-β1 (AB 1937), anti-α1 (AB 1934), anti-α3 (AB 1920) and anti-α5 (AB 1928) obtained from Chemicon (Temecula, CA, U.S.A.). The polyclonal antibodies against Shc (06-203) and the IGF-IR were purchased from Biomol (Hamburg, Germany). Polyclonal anti-phospho-p42/p44 Erk antibody was purchased from Promega (Mannheim, Germany). Polyclonal antibody against the β1 cytoplasmic domain was provided by Dr. F. G. Giancotti (Cellular Biochemistry and Biophysics Program, Memorial Sloan-Kettering Cancer Center, New York, NY, U.S.A.) and has been described previously [32]. The monoclonal antibody against integrin β1 (4B4) was purchased from Coulter (Hialeah, FL, U.S.A.), and those against integrins α1 (FB12), α3 (P1B5), α5 (P1D6), αv (VNR139) were from Chemicon. All of these antibodies recognize human integrins. The monoclonal antibodies against α-actinin (MAB 1682), vinculin (MAB 1674), paxillin (MAB 3060), FAK (MAB 2156) and anti-phosphotyrosine PY 20 (MAB 3080) were purchased from Chemicon. The monoclonal antibody against Grb2 (05-372) was purchased from Biomol. The monoclonal antibody against Shc (S52420) was from Transduction Laboratories (Hamburg, Germany). The anti-MHC monoclonal antibody W6.32 reacts with human and rat cells [33]. Secondary antibodies conjugated with alkaline phosphatase were purchased from Boehringer Mannheim, Mannheim, Germany.

Collagen types I and II, cytochalasin D, trypsin, collagenase, poly-L-lysine and *Staphylococcus aureus* cells were purchased from Sigma (München, Germany). IGF-I was purchased from Biomol. Pronase was from Boehringer Mannheim.

Methods

Chondrocyte culture

Human articular cartilage slices (from femoral heads obtained during joint-replacement surgery for femoral neck fractures) were collected in Ham's F-12 medium. Cartilage slices were rinsed with Hanks solution and digested with 1% Pronase (from *Streptomyces griseus*, 7000 units/g) in Hanks solution containing 5% (v/v) fetal calf serum for 2 h at 37 °C and then with 0.2% (v/v) collagenase (from *Clostridium histolyticum*, 0.15 unit/mg) in the same solution for 4 h at 37 °C. After rinsing in growth medium, a single-cell suspension was obtained by repeated pipetting and separation from undissolved tissue fragments using a nylon mesh with a pore width of 80 μm. Cells were sedimentated by centrifugation at 6000 ***g***, rinsed twice in growth medium [Ham's F-12/Dulbecco's modified Eagle's medium (1/1)/10% fetal calf serum/25 μg/ml ascorbic acid/50 μg/ml gentamycin], and resuspended in growth medium at 2×10^6/ml. The cells were cultured in alginate beads as described in detail by Shakibaei and de Souza [34]. To dissolve the alginate for subsequent separation of the cells, the alginate beads were placed in 55 mM sodium citrate in 0.15 M NaCl.

Measurement of collagen type II adherent chondrocytes in cultures with or without IGF-I treatment

Glass coverslips coated with collagen type II (500 μg/ml in 0.02 M acetic acid at 4 °C overnight) were washed three times with PBS, and then incubated with serum-free medium at 37 °C for 1 h before use. Human chondrocytes isolated from alginate beads were washed three times with serum-free medium. After counting, the cells were diluted to 1.5×10^6/ml in serum-free medium, cultured on prepared coverslips for 30 min and then either stimulated with IGF-I (100 ng/ml) or left untreated for the indicated times. After washing, the attached chondrocytes were fixed with 1% glutaraldehyde for 5 min and air-dried. The cultures were investigated after 1, 3, 5, 7, 9, 11, 13 and 15 h by light microscopy. The number of cells was determined by scoring cells from five different microscopic fields. These assays were performed in triplicate and the results are provided as mean values $\pm$ S.D. from three independent experiments.

Chondrocyte adhesion, immunoprecipitation and immunoblotting of integrins

Tissue-culture dishes were coated with collagen types I, II and poly-L-lysine (500 μg/ml in 0.02 M acetic acid) and monoclonal antibodies (5 μg/ml) at 4 °C overnight, washed three times with PBS, and then incubated with serum-free medium at 37 °C for 1 h before use. Human chondrocytes were harvested from alginate cultures and washed three times with serum-free medium. After counting, the cells were diluted to 1.5×10^6/ml in serum-free medium, plated on dishes coated with collagen types I, II, poly-L-lysine, anti-integrin β1 and anti-MHC antibodies and then treated with IGF-I (100 ng/ml) for 1 h or left untreated. In some experiments, chondrocytes were pretreated with cytochalasin D (0.1 μg/ml) for 30 min before exposure to IGF-I. After rinsing with PBS, cells were extracted with lysis buffer [50 mM Tris/HCl, pH 7.2/150 mM NaCl/1% (v/v) Triton X-100/1 mM sodium orthovanadate/50 mM sodium pyrophosphate/100 mM sodium fluoride/0.01% (v/v) aprotinin/4 μg/ml pepstatin A/10 μg/ml leupeptin/1 mM PMSF] on ice for 30 min. Insoluble material was removed by centrifugation at 10000 ***g*** for 30 min. Lysates were stored at -70 °C until use. For immunoblotting, equal amounts of total proteins were separated on 10% or 7.5% polyacrylamide gels by SDS/PAGE under reducing conditions. For immunoprecipitation, the extracts were precleared by incubation with 25 μl of normal rabbit IgG serum or normal mouse IgG serum and *S. aureus* cells, incubated with primary antibodies diluted in wash buffer (0.1% Tween 20/150 mM NaCl/50 mM Tris/HCl, pH 7.2/1 mM $CaCl_2$/1 mM $MgCl_2$/1 mM PMSF) for 2 h at 4 °C, followed by *S. aureus* cells for 1 h at 4 °C. Control immunoprecipitations were performed by incubating the samples with rabbit anti-mouse IgG alone. *S. aureus* cells were washed five times with wash buffer and once with 50 mM Tris/HCl, pH 7.2, followed by boiling in SDS/PAGE sample buffer. For immunoblotting, samples were separated by SDS/PAGE under reducing conditions. Proteins were transferred for 80 min at 120 V on to nitrocellulose (Schleicher & Schüll, Dassel, Germany) using a transblot electrophoresis apparatus (Mini Trans Blot™, Bio-Rad Laboratories, Richmond, VA, U.S.A.). Membranes were blocked with 5% (w/v) skimmed-milk powder in PBS/0.1% Tween 20 overnight at 4 °C and incubated with primary antibodies diluted in blocking buffer for 1 h at room temperature. After five washes in blocking buffer,

membranes were incubated with alkaline-phosphatase-conjugated secondary antibody diluted in blocking buffer for 30 min at room temperature. Membranes were finally washed five times in blocking buffer, twice in 0.1 M Tris, pH 9.5, containing 0.05 M $MgCl_2$ and 0.1 M NaCl; specific binding was detected using Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (*p*-toluidine salt; Pierce, Rockford, IL, U.S.A.) as substrates and quantfied by densitometry. Protein determination was done with the bicinchoninic acid system (Pierce) using BSA as a standard.

RESULTS

Effects of IGF-I on chondrocyte adhesion to collagen type II

We have previously reported that, in contrast with chondrocytes cultured on collagen type I and plastic, chondrocytes grown on collagen type II maintain their rounded phenotype and synthesize collagen type II. The interaction between chondrocytes and collagen type II was largely inhibited by anti-β1 integrin antibodies [5]. To obtain ligation of integrins in the absence of any other stimulation caused by serum growth factor, human chondrocytes, which express the IGF-I receptor [35], were cultured on collagen type II-coated Petri dishes, serum starved, and then either stimulated with IGF-I or left untreated. The chondrocytes grown on collagen type II with or without IGF-I always exhibited a round to oval shape and numerous small ridge-like or cuspoidal surface processes from the beginning of cultivation onwards. After plating, the number and density of the chondrocytes cultivated on collagen type II and in the absence of IGF-I was significantly lower than for those cultivated on collagen type II and treated with IGF-I. There is a loss of cells from the collagen with time in the absence of IGF-I. After a culture period of 3, 5, 7, 9, 11, 13 and 15 h, the total number of untreated chondrocytes grown on collagen type II was reduced by about 15 % ($P = 0.015$), 27 % ($P = 0.007$), 28 % ($P = 0.014$), 41 % ($P = 0.006$), 53 % ($P = 0.002$), 54 % ($P = 0.009$) and 59 % ($P = 0.005$) compared with those cultivated on collagen type II and in the presence of IGF-I (Figure 1). The experiments without collagen type II coating, even for the shorter incubations, will not be considered further in this study, since these resulted in either a mixed population or a pure population of fibroblast-like cells [5]. These results indicate a specific positive effect of collagen type II and IGF-I, but not other substrates (collagen type I, plastic), on the stabilization of chondrocyte differentiation, survival and adhesion.

Characterization of intracellular protein tyrosine phosphorylation in response to collagen type II adhesion and/or exposure to IGF-I in human chondrocytes

To investigate the hypothesis that integrin-dependent intracellular signalling proteins, influenced by IGF-I in chondrocytes, require the interaction of integrins with specific cartilage matrix proteins, serum-starved human chondrocytes were grown on collagen types I and II or poly-L-lysine in the presence or absence of IGF-I for 1 h. Some chondrocytes cultured on collagen type II were pretreated with cytochalasin D for 30 min before stimulation with IGF-I. Tyrosine phosphorylation of intracellular proteins was revealed by immunoblotting with anti-phosphotyrosine (p-Tyr) antibodies. As shown in Figure 2, the adhesion of untreated chondrocytes to collagen type II resulted in tyrosine phosphorylation of proteins with apparent molecular masses of 200 kDa, 190 kDa, 125 kDa, 100 kDa and 80–40 kDa. Furthermore, adhesion of IGF-I-treated chondrocytes to collagen type II revealed tyrosine phosphorylation of almost the same set of proteins, but was higher than that of those cultivated on collagen type II not treated with IGF-I. No significant tyrosine phosphorylation of proteins could be shown in chondrocytes plated on collagen type I or on poly-L-lysine with or without exposure to IGF-I, suggesting that the phosphorylation of signalling proteins was induced by binding to specific matrix components. Furthermore, we examined the effects of cytochalasin D, an inhibitor of actin filament polymerization, on integrin-mediated tyrosine phosphorylation in chondrocytes. Interestingly, no detectable phosphorylation of proteins was observed in chondrocytes cultured on collagen type II pretreated with cytochalasin D, even after exposure to IGF-I (results not

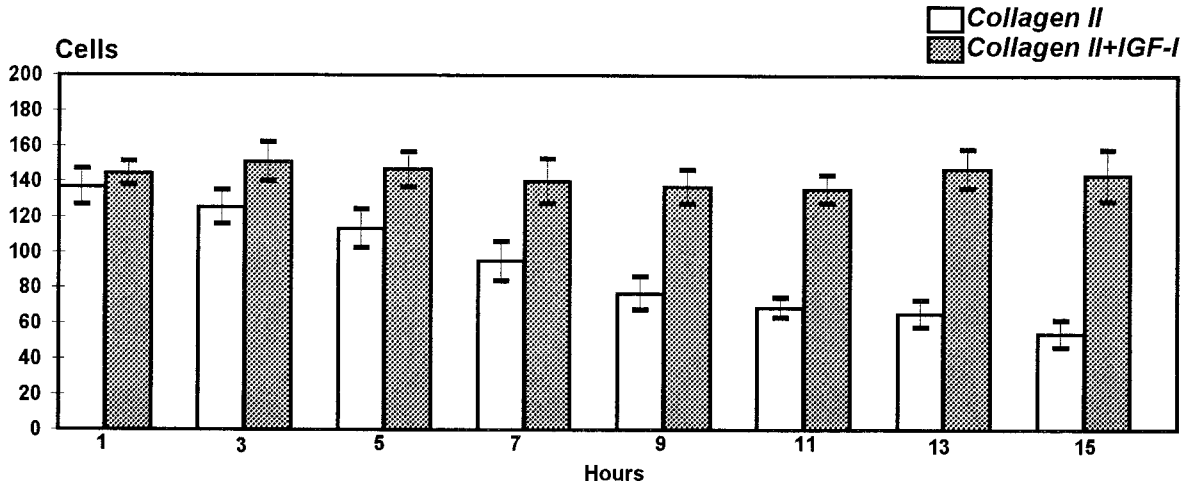

Figure 1 Effects of IGF-I on chondrocyte adhesion to collagen type II

Serum-starved human chondrocytes were cultured on glass coverslips coated with collagen type II, and then either treated with IGF-I (100 ng/ml) or left untreated in serum-free medium for 15 h. The adherent chondrocytes were quantified every 1 h by scoring cells from five different microscopic fields. Means ± S. D. from three independent experiments are indicated.

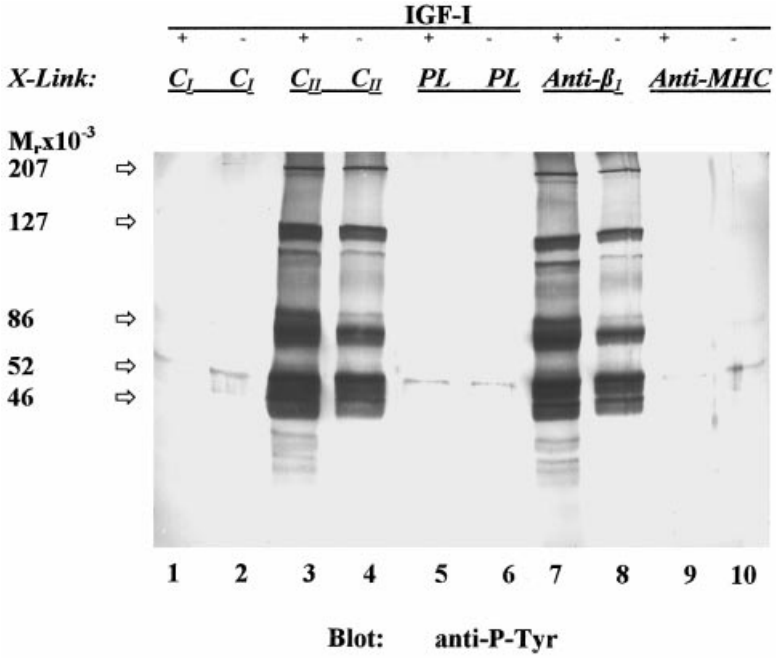

Figure 2 Tyrosine phosphorylation induced by engagement of extracellular matrix components and integrins

Serum-starved human chondrocytes were plated on dishes coated with collagen types I (C_I), II (C_{II}), poly-L-lysine (PL), anti-β1 integrin and anti-MHC antibodies and either treated with IGF-I (100 ng/ml) or left untreated in serum-free medium for 1 h. Equal amounts (50 μg of protein per lane) of total proteins were separated by SDS/PAGE (7.5% gel) and analysed by immunoblotting with anti-P-Tyr antibody. Chondrocytes were cultivated on: lane 1, collagen type I, exposed to IGF-I; lane 2, collagen type I, without IGF-I; lane 3, collagen type II, exposed to IGF-I; lane 4, collagen type II, without IGF-I; lane 5, poly-L-lysine, exposed to IGF-I; lane 6, poly-L-lysine, without IGF-I; lane 7, anti-β1 integrin antibodies, exposed to IGF-I; lane 8, anti-β1 integrin antibodies, without IGF-I; lane 9, anti-MHC antibodies, exposed to IGF-I; lane 10, anti-MHC antibodies, without IGF-I. Results shown are representative of three independent experiments.

shown), suggesting that cytoskeleton proteins are necessary for linking integrin receptors with signalling proteins in this signalling process [36]. Taken together, these results suggest that: (1) most of the effect on tyrosine phosphorylation appears to be due to collagen type II; and (2) the stimulation of chondrocytes by IGF-I induces an increase in the level of tyrosine phosphorylation.

To clarify further the role of β1 integrins in this process, chondrocytes were cultured either on dishes coated with anti-β1 integrin (4B4), to cluster the β1 integrin on the chondrocyte surface, or on dishes coated with anti-MHC for 1 h. Western blot analyses of chondrocytes cultured on collagen types I or II, poly-L-lysine, anti-β1 integrin and anti-MHC antibodies were performed an a single blot to minimize differences in technique. As shown in Figure 2, adhesion of chondrocytes to anti-β1 integrin antibodies, but not to anti-MHC antibodies, led to tyrosine phosphorylation of the same set of proteins found in collagen type II-adherent chondrocytes. Significant differences were found with regard to stimulated signalling proteins between chondrocytes with or without exposure to IGF-I. Densitometric analysis of a representative experiment performed in triplicate from adhesion of IGF-I-treated chondrocytes to anti-β1 integrin antibodies showed that the relative tyrosine phosphorylation of proteins with apparent molecular masses of 200 kDa, 190 kDa, 125 kDa, 100 kDa and 80–40 kDa increased by 15% ($P = 0.013$), 94% ($P = 0.009$), 35% ($P = 0.0001$), 73% ($P = 0.0007$), 58% ($P = 0.006$), 19% ($P = 0.0034$), 24% ($P = 0.016$), 39% ($P = 0.024$), 97% ($P = 0.027$), 95% ($P = 0.0054$), compared with adhesion of untreated chondrocytes to anti-β1 integrin antibodies. Taken together, these findings suggest that clustering of β1 integrins with antibody activates signalling pathways similar to those generated by chondrocyte adhesion to collagen type II, suggesting that the β1 integrin receptor is one of the major receptors mediating chondrocyte–collagen type II interaction. On the other hand, we cannot exclude the possibility that other integrin receptors or other non-integrin cell surface receptors, such as heparan sulphate proteoglycans [37], participate in this event.

To examine whether chondrocyte adhesion to collagen type II and treatment with IGF-I induces integrin phosphorylation, phosphotyrosine-containing proteins were immunoprecipitated from total lysates and probed with antibodies specific for β1, α3, α5 and αv integrins. The results revealed that the adhesion of chondrocytes to collagen type II and exposure to IGF-I does not cause tyrosine phosphorylation of integrins (results not shown).

The cytoplasmic domain of β1 integrin interacts physically with activated intracellular signalling proteins

To examine whether integrins can interact with FAK and other focal adhesion proteins, including α-actinin, vinculin and paxillin, in human chondrocytes adhering to collagen, co-immunoprecipitation assays were performed. Serum-starved chondrocytes were cultured on collagen types I and II, and then either treated with IGF-I or left untreated. After immunoprecipitation with anti-β1 integrin antibodies, the samples were probed by immunoblotting with anti-FAK, anti-paxillin, anti-vinculin or anti-α-actinin. The results indicated that β1 integrins interact with FAK, vinculin, paxillin and α-actinin in chondrocytes adhering to collagen type II, but not to collagen type I (Figure 3). The interaction of β1 integrins with focal adhesion components is enhanced in chondrocytes treated with IGF-I. Densitometric analysis of a representative experiment, performed in triplicate from adhesion of IGF-I-treated chondrocytes to collagen type II,

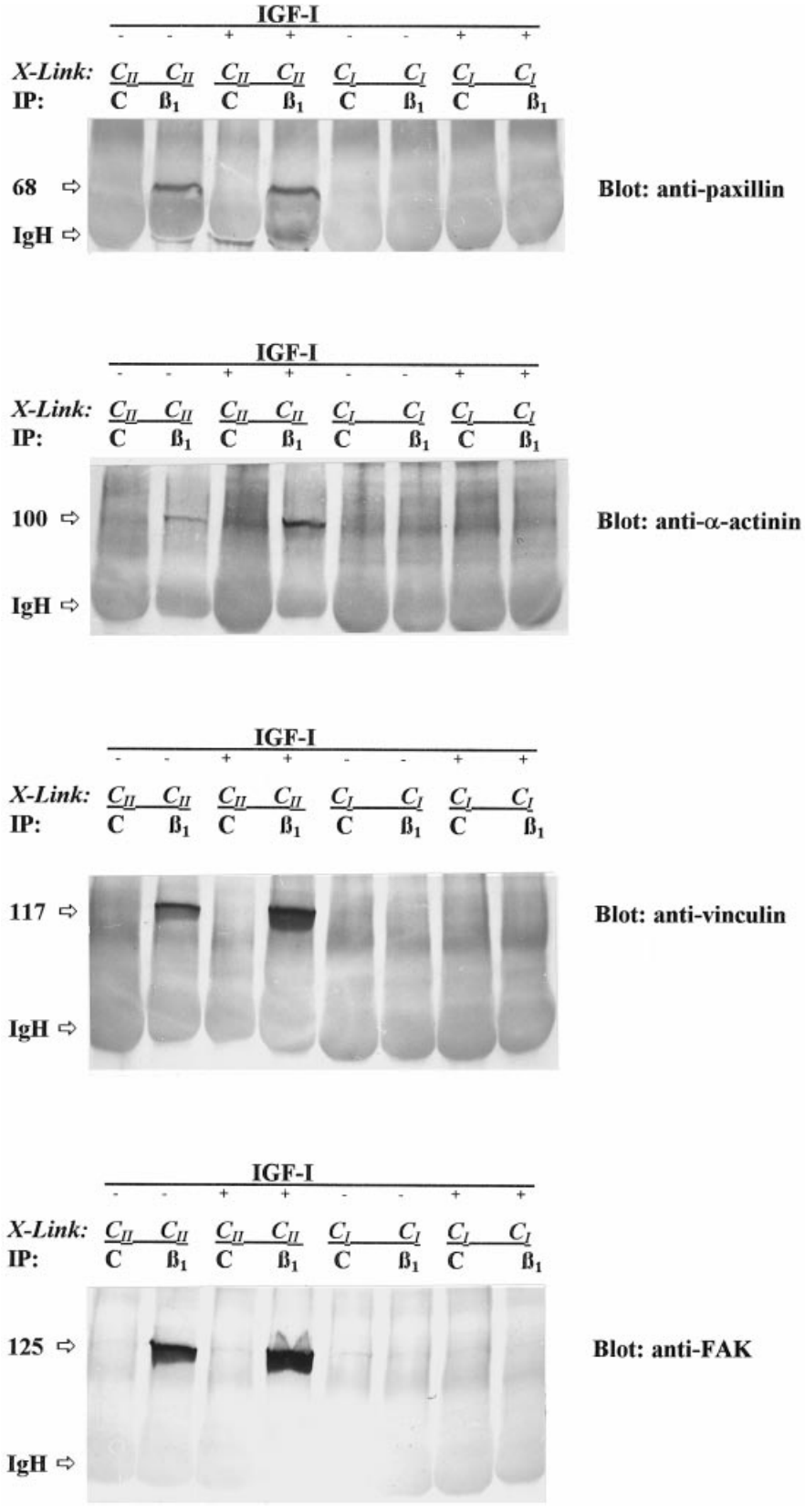

Figure 3 Interaction of the cytoplasmic domain of β1 integrin with intracellular signalling proteins

Serum-starved human chondrocytes were plated on dishes coated with collagen type II (C_{II}) or collagen type I (C_I) and then either treated with IGF-I (100 ng/ml) or left untreated for 1 h. Bound chondrocytes were lysed and immunoprecipitated (IP) with anti-β1 cytoplasmic domain (β_1) antibody and normal IgG serum, control (C). Immunoprecipitates were separated by SDS/PAGE (7.5% gel) and analysed by immunoblotting with anti-paxillin, anti-α-actinin, anti-vinculin and anti-FAK. Results shown are representative of three independent experiments. IgH, immunoglobulin heavy chain.

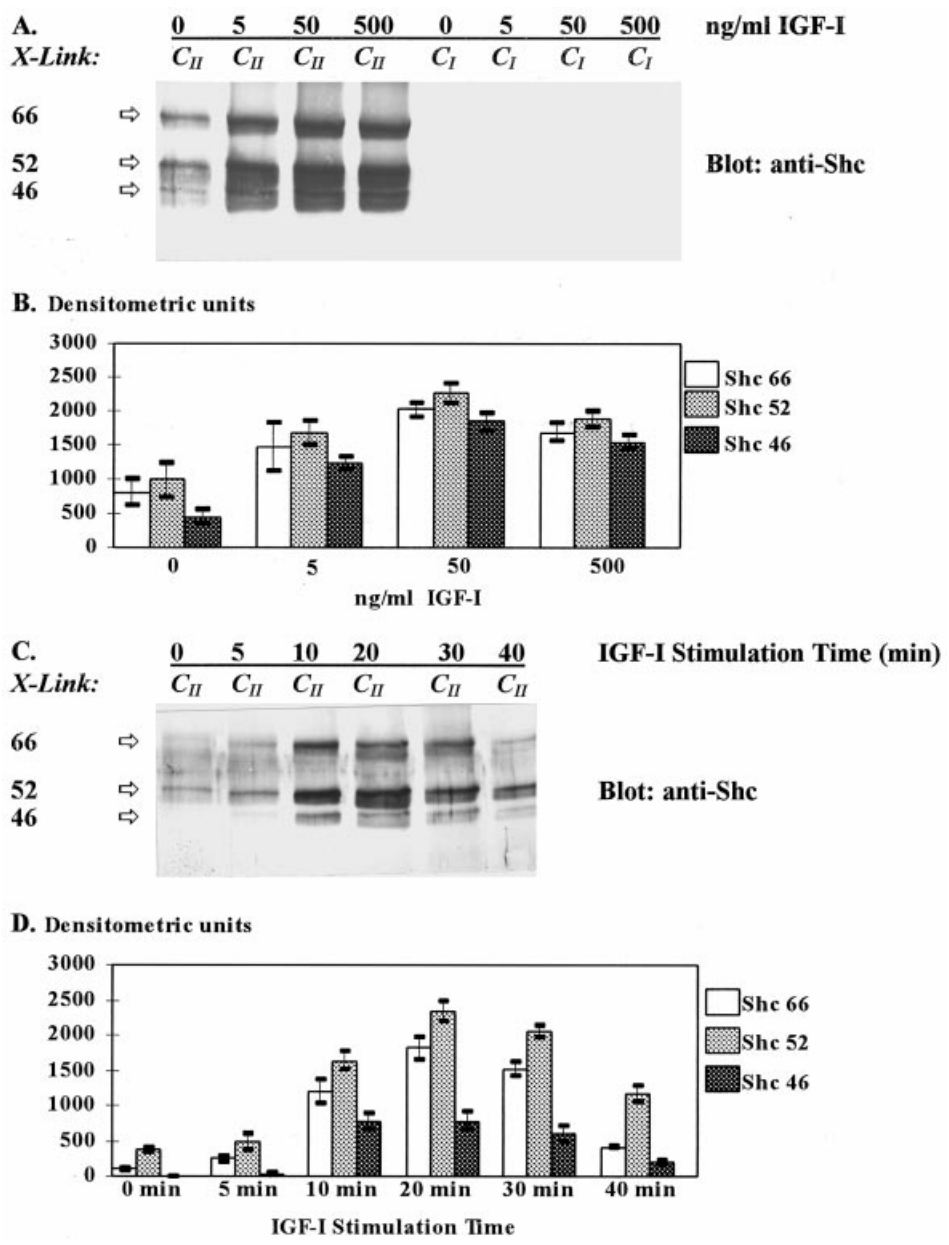

Figure 4 Dose- and time-dependence of IGF-I-mediated increase in expression of Shc adaptor proteins

Serum-starved human chondrocytes were plated on dishes coated with collagen type II (C_{II}) or collagen type I (C_I) and then treated with various concentrations of IGF-I: 0, 5, 50 and 500 ng/ml for 1 h (**A**). The bound chondrocytes were lysed and equal amounts of total protein were separated by SDS/PAGE (7.5% gel) and analysed by immunoblotting with anti-Shc antibodies. Densitometric quantification (**B**) demonstrates that prolonged peak Shc isoform expression occurs at 50 ng/ml IGF-I. (**C**) Time course of Shc isoform expression in chondrocytes plated on dishes coated with collagen type II. Cells were stimulated with 50 ng/ml IGF-I. Densitometric quantification (**D**) demonstrates that prolonged peak Shc isoform expression occurs between 10 min and 30 min. Values are means $\pm$ S.D. of a representative experiment performed in triplicate. Results shown are representative of three independent experiments.

showed that the relative paxillin expression co-immunoprecipitated with β1 integrin increased by 33% ($P = 0.0069$), with α-actinin by 64% ($P = 0.003$), with vinculin by 78% ($P = 0.013$) and with FAK by 36% ($P = 0.0082$) compared with adhesion of untreated chondrocytes to collagen type II.

Adaptor protein Shc expression is increased in response to IGF-I stimulation

Proteins that contain SH2 domains, such as Shc proteins (Shc has three isoforms, p66, p52 and p46), are implicated in the protein–protein interactions detected in integrin-mediated signalling pathways [14]. Chondrocyte adhesion to anti-β1 integrin antibodies or collagen type II revealed tyrosine phosphorylation of proteins with molecular masses of 66 kDa, 52 kDa and 46 kDa (Figure 2). To test this notion, we examined the role of Shc proteins in chondrocytes cultured on collagen types I and II with or without exposure to IGF-I. In addition, to verify that the IGF-I-induced increase in Shc expression is dose-dependent, serum-starved human chondrocytes cultured on collagen types I and II were treated with various concentrations of IGF-I (0-500 ng/ml), lysed, and immunoblotted with anti-Shc antibodies.

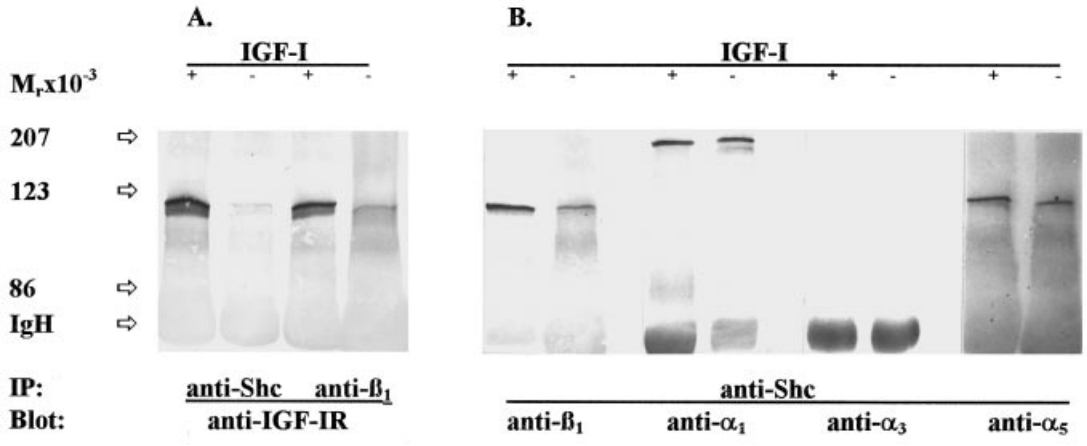

Figure 5 Binding of the adaptor protein Shc to activated IGF-IR as well as to integrin receptors and binding of IGF-IR to integrin receptors in chondrocytes

Serum-starved human chondrocytes were plated on to dishes coated with collagen type II and then treated with IGF-I (100 ng/ml) or left untreated for 1 h. Equal amounts of total proteins were lysed and immunoprecipitated using 2 μg of anti-Shc and anti-β1 integrin antibodies. Immunoprecipitates (IP) were separated by SDS/PAGE (7.5% gel) and analysed by immunoblotting with either anti-IGF-IR antibodies, or anti β1-, α1, α3 or α5 integrin antibodies. Results are representative of three independent experiments. IgH, immunglobulin heavy chain.

The results showed a marked dose-dependent increase in expression of all three Shc isoforms (p66, p52, p46) in chondrocytes cultured on collagen type II, but not on collagen type I (Figure 4A). Densitometric analysis of the results indicated a significant increase in the expression of all Shc isoforms in response to as little as 5 ng/ml IGF-I, and maximal expression levels in response to 50 ng/ml IGF-I (Figure 4B). An increase in the expression of all three Shc isoforms was apparent within 10 min of exposure to 50 ng/ml IGF-I in chondrocytes cultured on collagen type II, but not on collagen type I, and maximum expression was observed by 20 min of treatment (Figures 4C and 4D). These results suggest that all three Shc isoforms participate in IGF-I signalling and that a switching on of Shc expression occurs 10 min after initiation of IGF-I stimulation. Taken together, these findings indicate that the expression of Shc isoforms by IGF-I is dose- as well as time-dependent and that this stimulation requires the ligation of integrin to collagen type II.

Adaptor protein Shc binds to activated IGF-IR as well as to integrin receptors and IGF-IR binds to integrin receptors in chondrocytes

Serum-starved human chondrocytes were cultured on collagen type II in the presence or absence of IGF-I, lysed, and immunoprecipited with anti-Shc and anti-β1 integrin antibodies. The anti-Shc and anti-β1 integrin immunoprecipitates were then immunoblotted with anti-IGF-IR-antibodies and anti-Shc immunoprecipitates were immunoblotted with anti-β1, -α1, -α3 and -α5 integrin antibodies. Several studies have suggested that chondrocytes express IGF-I receptors [35] and the integrins β1, α1, α3 and α5 [3–5,38,39]. Figure 5(A) shows that IGF-IR co-immunoprecipitated with Shc proteins and β1 integrin receptor from IGF-I-stimulated cultures. Shc and β1 integrin proteins isolated from unstimulated cultures contained very low levels of IGF-IR. Densitometric analysis of a representative experiment, performed in triplicate from adhesion of IGF-I-treated chondrocytes to collagen type II, showed that the relative IGF-IR expression co-immunoprecipitated with Shc proteins increased by 95% ($P = 0.0033$) and with β1 integrin receptor by 79% ($P = 0.0002$), compared with adhesion of untreated chondrocytes to collagen type II. Anti-Shc immunoprecipitates, with or without IGF-I-stimulation, revealed that the β1 , α1 and α5 integrins, but not α3 integrins, co-immunoprecipitate with Shc proteins (Figure 5B). Shc proteins isolated from unstimulated cultures contained low levels of β1, α1 and α5 integrins. Densitometric analysis of a representative experiment, performed in triplicate from adhesion of IGF-I-treated chondrocytes to collagen type II, showed that the relative β1 integrin expression co-immunoprecipitated with Shc proteins increased by 54% ($P = 0.023$), α1 integrin 11% ($P = 0.0096$) and α5 integrin 23% ($P = 0.0008$), compared with adhesion of untreated chondrocytes to collagen type II. In addition, the same results were obtained when IGF-I-stimulated chondrocytes were immunoprecipitated with anti-IGF-IR, anti-β1, α1, α3 and α5 integrin antibodies and then immunoblotted with anti-Shc antibody, revealing that the IGF-IR, β1, α1 and α5 integrins co-immunoprecipitated with Shc proteins (results not shown). Taken together, these findings show that Shc adaptor proteins associate with activated IGF-IR in response to IGF-I activation, as well as with distinct integrin receptors. Furthermore, these results suggest that integrin receptors co-immunoprecipitate with the IGF-IR and indicate that they are part of the same complexes.

Association of Shc proteins with the adaptor protein Grb2 and activated Erk in chondrocytes

To define possible downstream signalling proteins in chondrocytes cultured on collagen type II, we examined whether Shc protein associates with the adaptor protein Grb2-associated son of sevenless and subsequently activates the Ras-MAP kinase pathway. To this end, serum-starved human chondrocytes were cultured on collagen type II in the presence or absence of IGF-I, lysed, and immunoprecipitated with anti-Shc antibodies. The anti-Shc immunoprecipitates were then immunoblotted with anti-Grb2 and anti-phospho-Erk antibodies. Since Erk activation requires phosphorylation of both threonine and tyrosine residues [40], stimulated Erk can be detected using the antibody specific for dually phosphorylated Erk. Anti-Shc immunoprecipitates from IGF-I-stimulated or from unstimulated cultures revealed that the Grb2 adaptor protein and activated Erk1 and Erk2 co-

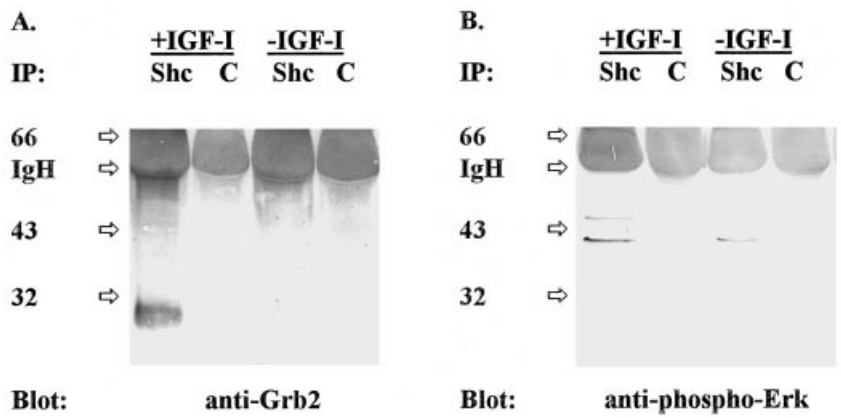

Figure 6 Association of Shc proteins with the adaptor protein Grb-2 and activated Erk in chondrocytes

Serum-starved human chondrocytes were plated on to dishes coated with collagen type II and then treated with IGF-I (100 ng/ml) or left untreated for 1 h. Equal amounts of total proteins were lysed and immunoprecipitated using 2 μg of anti-Shc antibody or normal IgG-serum, control (C). Immunoprecipitates were separated by SDS/PAGE (10% gel) and analysed by immunoblotting with anti-Grb2 (23 kDa) and anti-activated Erk1 (44 kDa) and Erk2 (42 kDa) antibodies. Results are representative of three independent experiments. IgH, immunoglobulin heavy chain.

immunoprecipitated with Shc proteins. However, IGF-I induced a marked increase in the amount of Shc-associated Grb2 adaptor protein/Erk1 and Erk2 (Figure 6). Densitometric analysis of a representative experiment, performed in triplicate from adhesion of IGF-I-treated chondrocytes to collagen type II, showed that the Shc proteins co-immunoprecipitated with Grb2 adaptor protein increased by 97.5% ($P = 0.008$), with activated Erk1 by 67% ($P = 0.016$) and with Erk2 by 59% ($P = 0.0024$), compared with adhesion of untreated chondrocytes to collagen type II. Taken together, these results indicate that in chondrocytes adhering to collagen type II and exposed to IGF-I, activated IGF-IR and distinct integrin receptors induce Shc–Grb2 complex formation, which activates the Ras–MAP kinase pathway.

DISCUSSION

The major findings in this study are as follows: (1) when chondrocytes are cultured on collagen type II and exposed to IGF-I, cultures reveal a higher density of chondrocyte adhesion compared with cultures in the absence of IGF-I; (2) tyrosine phosphorylation of intracellular integrin-dependent signalling proteins is present in chondrocytes plated on anti-β1 integrin antibodies or on collagen type II, and is stimulated after exposure to IGF-I; (3) no significant tyrosine phosphorylation of intracellular proteins can be shown in chondrocytes plated on collagen type I or on poly-L-lysine, with or without exposure to IGF-I; (4) expression of the docking protein Shc is increased in chondrocytes bound to collagen type II or integrin antibodies, and its level is increased when IGF-I is added; (5) Shc protein binds to either IGF-IR or distinct integrin receptors, suggesting a collaboration between integrins and growth factors in a common/shared biochemical signalling pathway; and (6) Shc then associates with Grb2 and with phospho-Erk, leading to activation of the Ras–MAP kinase signalling pathway.

We found the increased phosphorylation of intracellular signalling proteins induced by IGF-I in chondrocytes cultured on collagen type II occurred in response to β1 engagement, because the same effect was observed in chondrocytes cultured on β1 integrin antibody. The IGF-I changed the ability of chondrocytes to adhere to collagen type II, but not to collagen type I or poly-L-lysine. Furthermore, we could show that the β1 integrin co-immunoprecipitated with IGF-IR in chondrocytes cultured on collagen type II and treated with IGF-I, suggesting that this association could play an important role in the regulation of chondrocyte adhesion to collagen type II in a synergistic manner. In fact, Vuori and Ruoslahti [23] have reported that IRS-1 binds to $\alpha v\beta 3$ integrins in cells stimulated by insulin. However, the mechanism by which integrins and specific growth factors, such as IGF-I, regulate chondrocyte functions and differentiation are at present not fully understood.

We have also shown that IGF-I markedly stimulates the intracellular signalling proteins localized at focal adhesions and adaptor proteins, such as Shc isoforms (p66, p52 and p46), suggesting that all Shc isoforms play an important role in IGF-I signalling in human chondrocytes. In contrast to this finding, it has been shown that insulin or IGF-I stimulates activation of p52 and p66 or p52 and p46 in other cell types, but not all Shc isoforms simultaneously [16,41,42]. The co-immunoprecipitation assay of Shc with integrins strongly suggested that Shc has its effect on downstream signal transduction by interacting with the distinct cell surface β1, α1, α5 integrins, but not with the α3 integrin. In addition, these results confirm the observation that the interaction of the $\alpha 1\beta 1$, $\alpha 5\beta 1$ and $\alpha v\beta 3$ integrins, but not $\alpha 2\beta 1$, $\alpha 3\beta 1$ and $\alpha 6\beta 1$ with Shc is mediated by the membrane-proximal region or transmembrane domains of the α subunit [14]. On the other hand, co-immunoprecipitation of IGF-IR with Shc and with β1 integrin showed that IGF-IR associates with Shc as well as with integrin receptor. This physical association was markedly increased in the presence of IGF-I. In fact, it has been reported that upon stimulation of Rat-1 cells with epidermal growth factor, the Shc adaptor proteins become phosphorylated and binds to activated epidermal growth factor receptors [14]. It seems that distinct integrin receptors and IGF-IR may have a common downstream target for their intracellular signalling pathways.

It is tempting to hypothesize that the integrins mediate collagen type II-induced differentiation and adhesion of chondrocytes by interacting with the growth factor that stimulates chondrocyte adhesion and activates IGF-IR, leading to the intracellular signalling required for stabilization of the chondrocyte phenotype. In fact, the colocalization of growth factor receptors and integrins in the focal adhesion contacts and their structural

vicinity has been described previously [30]. Furthermore, all these observations support the hypothesis that focal adhesion assembly may facilitate signalling via Shc, a common point for signal integration and potentiation between integrin and growth factor signalling pathways in chondrocytes.

Our results show that IGF-I treatment of chondrocytes plated on collagen type II caused a significant increase in the expression of Shc and a sustained association of Shc with Grb2, Erk1 and Erk2. It may be suggested that the association of Shc with Grb2 is responsible for the fact that IGF-I activates the MAP kinase pathway. Shc has been demonstrated to be an Src homology 2-phosphotyrosine-binding domain docking protein which regulates signal transduction [13]. Several lines of evidence suggest that activation of Shc by growth factors and integrins and its association with Grb2 adaptor protein activates the Ras signalling pathway [14,43]. Our results provide evidence that Shc expression may become increased independently in chondrocytes after integrin binding to collagen type II, with or without stimulation with IGF-I, and that in the presence of IGF-I Shc expression is markedly increased.

Interestingly, the level of Grb2, Erk1 and Erk2 binding to Shc in chondrocytes cultured on collagen type II was very low, but the level of Grb2, Erk1 and Erk2 bound to Shc in the same cells in the presence of IGF-I was significantly increased. Therefore, it is likely that Shc may be a common point in these two signal-transduction pathways and that a summation of signals may be responsible for the increasing level of Shc and Grb2/Erk expression. Miyamoto et al. [25] have shown that the synergistic interaction between integrins and growth factor receptors phosphorylates MAP kinases, which control a variety of cell functions, and that this collaboration is only possible if the integrins are already aggregated and occupied by a ligand.

The collaborative effect of integrins on the IGF-IR signalling pathway in chondrocytes may be one of the major molecular mechanisms controlling cytoskeletal organization, cell differentiation and cell survival. To verify this, it will be necessary to work out further details of the integrin-stimulated signalling cascade in chondrocytes cultivated on different types of extracellular matrix proteins, the role of integrins in regulating gene expression in chondrocytes and their collaboration with growth factors.

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