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## Altered expression of integrins in RSV-transformed chick epiphyseal chondrocytes

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### Abstract

Chondrocytes have been shown to express both in vivo and in vitro a number of integrins of the beta1-, beta3- and beta5-subfamilies (Biorheology 37 (2000) 109). Normal and v-Src-transformed chick epiphyseal chondrocytes (CEC) display different adhesion properties. While normal CEC with time in culture tends to increase their adhesion to the substrate by organizing focal adhesions and actin stress fibers, v-Src-transformed chondrocytes display a refractile morphology and disorganization of actin cytoskeleton. We wondered whether the reduced adhesion and spreading of v-Src-transformed chondrocytes could be ascribed to changes in integrin expression and/or function. Integrin expression by normal CEC is studied and compared to v-Src-transformed chick chondrocytes, using monoclonal and polyclonal antibodies to integrins alpha- and beta-chains. We show the presence of alpha1-, alpha3-, alphav-, alpha6-, beta1- and beta3-chains on CEC, with very low levels of alpha2- and alpha5-chains. Alphav chain associates with multiple beta subunits in normal and transformed chondrocytes. With the exception of alpha1- and alpha2-chains, the levels of the integrin chains analyzed are higher in transformed chondrocytes as compared with normal chondrocytes. In spite of the increased levels of integrin expression, transformed chondrocytes exhibit loss of focal adhesion and actin stress fibers and low adhesion activity on several extracellular matrix constituents. These observations raise the possibility that, in addition to its effects on global pattern of integrin expression, v-Src can influence integrin function in chondrocytes.

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**Keywords:** Cell adhesion; Integrins; v-Src; Cell transformation

### 1. Introduction

The transforming protein of RSV, v-Src, is a cytoplasmic tyrosine kinase with constitutive activity [2–5]. v-Src-transformed cells exhibit several properties related to cell adhesion, including morphological changes, loss of anchorage dependent growth, reduction in number and size of focal adhesions, and decreased synthesis and deposition of extracellular matrix (ECM) [4–8].

Cell transformation by v-Src is mediated by the activation of multiple signaling pathways, including the phosphatidylinositol 3-kinase (PI3-kinase)/Akt and Ras/mitogen-activated protein (MAP) kinase pathways [9–14]. However, it is still unclear how the activation of these signaling pathways induces the extensive morphological cellular transformation, i.e. the reduction in number and size of focal adhesions and actin stress fibers, and the change from a well spread to an elongated spindle-shaped morphology, or even complete rounding and detachment. Regulation by v-Src of the Rho family of small GTPases, which control cytoskeletal organization, has been linked to morphological transformation [15,16]. Recently, it has been shown that the activation of MEK is required for the morphological changes and reorganization of the actin cytoskeleton induced by v-Src expression in rat fibroblasts [17]. The v-Src oncoprotein is translocated to integrin-linked focal adhesions, where its tyrosine

**Abbreviations:** CEC, chicken epiphyseal chondrocytes; CEF, chicken embryo fibroblasts; SR RSV A, Rous sarcoma virus, Schmidt–Ruppin strain, subgroup A; CEC-RSV, CEC transformed with a wt v-Src kinase; FN, fibronectin.

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kinase induces adhesion disruption and cell transformation [18,19]. v-Src causes extensive tyrosine phosphorylation of focal adhesions components, including the beta1 integrin subunit, talin, paxillin, Src and FAK [20–23]. Phosphorylated beta1 integrins interact less well with fibronectin via integrin extracellular domain or with talin via intracellular domain [22,24]. The phosphorylated integrin is located in podosomes [25], the dot-shaped spots where transformed cells adhere transiently to their matrix [26–30], to which several other focal adhesion components, including talin, are also redistributed upon transformation [31].

Integrins, a class of ECM receptors that transduce signals for cell growth, migration and differentiation, often display altered expression, function and localization in transformed cells [32–34]. Integrins are heterodimeric (one alpha and one beta subunit) transmembrane glycoproteins consisting of large globular extracellular domains that bind to specific ECM proteins and short cytoplasmic domains interacting with cytoskeletal proteins and signaling proteins inside the cell [35]. Chondrocytes are the only cell type present in cartilage and are surrounded by an abundant ECM consisting of several types of collagens (predominately types II, IX, XI, VI), proteoglycans and various glycoproteins [36]. Chondrocytes have been shown to express both in vitro and in situ a number of integrins of the beta1, beta3 and beta5 families [1], but the functional roles of chondrocyte integrins in normal or diseased cartilage have only recently come under study ([37–41]; for reviews see Refs. [1,42]). Primary cultures of embryonic chondrocytes have been widely used to study the regulation of chondrocyte functions. Initially isolated as cells growing in suspension, primary chick epiphyseal chondrocytes (CEC) attach and assume the typical polygonal shape [43]. With time in culture, monolayer chondrocytes spread out, assume a fibroblastic shape and dedifferentiate [44]. Dedifferentiation of chondrocytes occurring upon cell shape changes is associated with focal adhesion and stress fiber organization [27,45].

Upon transformation induced by RSV, polygonal CEC elongate and acquire a spindle-shaped morphology [43,46]. We have shown that the transformation of CEC by v-Src causes the disruption of the organized actin cytoskeleton and cellular focal adhesions and induces the formation of podosomes [27,47].

Here, we report on the expression of integrins in normal and RSV-transformed chondrocytes. We performed this study using also NA101, a chondrocyte derived line that stably expresses v-Src [48].

Normal and transformed chondrocytes display different adhesion properties. While normal chondrocytes tend to increase their adhesion to the substrate by organizing focal adhesions and actin stress fibers [27], v-Src-transformed chondrocytes display a refractile morphology, loss of focal adhesions and stress fibers [27,47,48]. We wondered whether a low integrin expression was associated with the reduced adhesion and spreading of transformed chondrocytes. We found instead that, with the exception of alpha1- and alpha2-

chains, the integrin chains analyzed are expressed in greater amounts in RSV-transformed chondrocytes as compared with the levels expressed by control CEC. Although v-Src promotes increased integrin expression in chondrocytes, the overall consequences of v-Src transformation are the loss of focal adhesions and actin stress fibers, leading to decreased attachment to several ECM proteins.

## 2. Materials and methods

### 2.1. Cell cultures

Embryonated chicken eggs (white Leghorn) were obtained from Lohmann Tierzucht GmbH, Cuxhaven, Germany. Chicken embryo fibroblasts—CEF—were grown in Eagle's MEM plus 4% calf serum, 1% chicken serum, 10% Triptose phosphate broth.

Primary epiphyseal chondrocytes were isolated from day 10 chicken embryo tibiae as previously described [43] with the following modifications. Tibiae were incubated for 1 h in a saline containing trypsin/collagenase, and the cells released after this incubation were discarded as they consisted mainly of perichondrial cells. Epiphysis were dissected, minced and incubated at 37 °C in fresh enzyme mixture; cells released by sequential enzymatic digestion from about 50 embryos were pooled and seeded at a low initial cell density (2000 cells/cm<sup>2</sup>) in Coon's modified F12 medium supplemented with 10% fetal calf serum. Floating cells were routinely collected and propagated once a week. The expression of the differentiated phenotype was monitored by analyzing the cell shape and the [<sup>3</sup>H]-labeled collagens.

Primary chick epiphyseal chondrocytes could grow either in suspension or in monolayer and express the cartilage phenotypic traits. Monolayer chondrocytes were studied as the normal counterparts of the transformed chondrocytes and used in the infection experiments. Viral infections were performed as previously reported with a clonal isolate of Schmidt-Ruppin strain of RSV (SR RSV A) [43].

### 2.2. Antibodies and reagents

Polyclonal antibodies against the cytoplasmic domain of beta1A and beta5 integrin subunits were generated as previously described [49,50]; polyclonal antibodies directed against the cytoplasmic domain of alpha1-, alpha3- and chicken alphav-subunits were previously described [51,52]; polyclonal antibodies directed against the cytoplasmic domain of chicken alpha2- [53] and alpha3-subunits [54] were generously provided by Dr. Reichardt.

The monoclonal antibodies B3D6 anti-chicken fibronectin [55], D71E2 anti-chicken alpha5 integrin subunit [56], JLA20 anti-chicken actin [57] were obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Biology, University of Iowa (Iowa City, IA).

The monoclonal antibody directed against the extracellular domain of chicken beta1 integrin, W1B10, was purchased

from Sigma [58]. The monoclonal antibody directed against the extracellular domain of the human and chicken beta3-chain was purchased from Transduction Laboratories (Nottingham, United Kingdom). The monoclonal antibodies directed against chicken alpha6-subunit [58], chicken alpha<sub>v</sub>, Chav-1, [59], against the alpha<sub>v</sub>-beta3 receptor, LM609 [60] and against alpha<sub>v</sub>beta5 [61] were purchased from Chemicon (Temecula, CA).

HRP-conjugated secondary antibodies, protein A- and protein G-agarose, and the ECL reagent were all from Santa Cruz Biotech (Santa Cruz, CA). Laminin, fibronectin and vitronectin were all obtained from Beckton Dickinson (Mountain View, CA). Collagen type I, collagen type II, fibrinogen, bovine serum albumin (BSA), fetal calf serum, molecular weight markers were all from Sigma (St. Louis, MO).

### 2.3. Adhesion experiments

Wells of 96-well microtiter plates (Costar/Corning) were coated with 20 µg/ml of purified fibronectin, laminin, vitronectin, osteopontin, or with 50 µg/ml of collagen types I or II by overnight incubation at 4 °C and post-coated with BSA for 1 h at 37 °C. Fifty thousand cells were placed in wells in 50 µl of DMEM medium. After 60' at 37 °C in 5% CO<sub>2</sub>, wells were washed with PBS and the number of adherent cells were determined by assaying for lysosomal *N*-acetyl-β-hexosaminidase activity [62].

### 2.4. Immunoprecipitation, SDS-PAGE, and Immunoblotting

Cells were washed twice and detergent extracted in a lysis buffer containing 0.5% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 2 mM EDTA, 10 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 0.15 U/ml trypsin inhibitory aprotinin, antipain 20 µg/ml, and 4 µg/ml pepstatin for 20 min at 4 °C and centrifuged at 14,000 × *g* for 20 min.

Protein concentration was determined in each cell extract by the Bio-Rad protein assay method. When cell extracts were analyzed, samples containing equal amounts of proteins were subjected to 7.5% SDS-PAGE under non-reducing conditions. Proteins were transferred to nitrocellulose (Schleicher and Schuell, Dassel, Gerusalem) using a Bio-Rad semidry apparatus (Munich, Germany) according to the manufacturer instructions. The blots were incubated overnight at 4 °C in 5% milk in Tris-buffered saline-Tween 20 (TBS-T: 150 mM NaCl, 20 mM Tris-HCl, pH 7.4, and 0.1% Tween), washed with TBS-T, and incubated 2 h with the indicated antibodies in TBS and 2.5% milk. The blots were washed three times with TBS-T, incubated 1 h with anti-mouse IgG peroxidase conjugate or with anti-rabbit IgG peroxidase conjugate and washed three times with TBS-T. The integrin subunits were visualized by the ECL detection method. Conditions of the development with the chemiluminescent substrate and exposure times were set to obtain a linear response.

Equal amounts of cell extracts were immunoprecipitated with the indicated antibodies, and immunocomplexes were bound to protein A- or protein G-agarose beads and recovered by centrifugation. Immune complexes were washed with lysis buffer containing 1% Chaps, once with the 50 mM Tris-HCl, pH 7.4 containing 0.5 M LiCl and three times with 10 mM Tris-HCl pH 7.4. Bound material was eluted by boiling beads in 2% SDS and separated on 7.5% SDS-PAGE in non-reducing conditions. After electrophoretic transfer, filters were processed for immunoblot analysis and then subjected to ECL detection method.

For metabolical labeling experiments, cells were incubated for 1 h in methionine-free F12 medium and then labeled for 6 h with 250 µCi/ml of [<sup>35</sup>S]-methionine (NEN 100 Ci/mmol). After cell extraction as described above, homogenates were incubated for 1 h with the non-immune serum, then with protein A-agarose and then centrifuged. Samples of labeled proteins containing equivalent amounts of cpm were then incubated overnight with the indicated antibodies and processed for immunoprecipitations using protein A- or protein G-agarose. Samples were subjected to SDS-PAGE on 6% acrylamide gels under non-reducing conditions for detection of protein complexes. After fluorography, gels were dried and exposed to Hyperfilm films (Amersham, Arlington Heights, IL).

## 3. Results

### 3.1. General characterization of control and RSV-transformed chondrocytes

Primary embryonic chondrocytes, obtained from chicken embryo epiphysis (CEC), grow in suspension or in monolayer, and express the cartilage collagens II and IX and the α<sub>1</sub>(I) collagen chain. With time in culture chondrocytes growing in suspension tend to attach and assume the typical polygonal morphology. Conversion from polygonal to suspension chondrocytes also occurs in young cultures. The phenotype of chondrocytes growing in monolayer is unstable, and the transition from polygonal to a spread fibroblastic shape has been observed upon subculture of monolayer chondrocytes from many species. Such transition is accompanied by a change in collagen expression from type II to type I collagen and stimulation of fibronectin synthesis [44,63]. Increased adhesion to the substrate of monolayer chondrocytes depends on cytoskeletal reorganization and alterations in cell-matrix interactions [27,45]. Thus, increased adhesion to the substrate of chondrocytes is associated with phenotypic instability. CEC growing in monolayer were used in this study as the normal counterparts of the transformed cells and were used in infection experiments, as well. To monitor the expression of the differentiated phenotype, cell shape and [<sup>3</sup>H]-labeled collagens were analyzed.

Upon transformation by RSV, CEC(RSV) acquire a spindle-shaped morphology [43]. pp60<sup>v-Src</sup> blocks the expression of cartilage ECM proteins and induces the synthesis

of type I collagen along with enhanced fibronectin expression [43]. Freshly transformed chondrocytes represent a cell system that is not easily controllable, because of the differences between experiments in the number of infected cells and v-Src levels obtained. Thus we therefore analyzed both the freshly transformed chondrocytes, CEC(RSV), and the immortalized NA101 cells derived from RSV-transformed chondrocytes [48]. Like freshly transformed chondrocytes, NA101 cells display a spindle-shaped morphology, and are able to grow in suspension in semisolid medium and in low serum conditions. In spite of repeated subcloning, it was soon evident that with time in culture NA101 cells started to synthesize lower and lower levels of collagen I and fibronectin [48]. NA101 cells tend to detach from the substrate, and become round and float in the medium. These data are consistent with reports showing that fibronectin and collagen I genes are negatively regulated by v-Src (reviewed in Ref. [64]).

We have shown that the transformation of chicken embryo chondrocytes by v-Src causes a dramatic and rapid redistribution of microfilaments [27,48]. The major feature was that F-actin-containing dots and rosettes resulting from the clustering of dots appeared upon transformation; these structures, podosomes, were identified as de novo formed adhesion sites and found to concentrate also  $\alpha$ -actinin and vinculin. Podosomes, which represent the main location of actin in freshly transformed cells, were present in NA101 cells and co-existed with some stress fibers [48].

Thus, while transformed chondrocytes display decreased spreading and adhesion, normal chondrocytes tend to increase their adhesion to the substrate by developing stress fibers and focal adhesions. Therefore, we asked whether the different adhesion properties displayed by normal and transformed chondrocytes correlate with different integrin expression.

### 3.2. Integrins expressed by normal and RSV-transformed chondrocytes

The levels of the accumulated integrins were measured in normal and RSV-transformed chondrocytes by immunoblot experiments. The alpha3chain was found to be present at higher levels in RSV transformed cells as compared with differentiated chondrocytes (Fig. 1A). The possibility that contaminating fibroblasts may be accounting for the alpha3 expression can be excluded as only normal chondrocytes growing in suspension are harvested and propagated. In all the preparations of chondrocytes tested, two bands by the anti-beta1 integrin antibody were detected. Besides the band in the  $M_r$  range of the mature form of beta1 subunit, the second one, with an apparent  $M_r$  below 116 kDa, could represent a degraded or a precursor form of the beta1-chain [65]. The expression of alpha1-chain declined in freshly transformed chondrocytes and could not be detected in NA101 cells, which have lost collagen I expression (Fig. 1B, due to the low levels of expression of the alpha1-chain, higher amounts of proteins were loaded on this gel).

We failed to detect significant expression of the alpha2 subunit either in control or in transformed chondrocytes by immunoblot experiments (not shown). Low levels of alpha5 expression were found in control chondrocytes; alpha5 levels were significantly higher in freshly transformed chondrocytes, and they declined in immortalized NA101 cells (Fig. 1C). Fibronectin levels undergo parallel changes as alpha5 levels do in control and transformed chondrocytes (Fig. 1C). alpha5beta1 is the high affinity receptor specific for fibronectin. Alpha5 expression is enhanced in freshly transformed and immortalized RSV-chondrocytes (Fig. 1D). The levels of the beta3 chain were significantly higher in transformed chondrocytes, as well (not shown). To determine whether alpha5 subunit associates with beta1, alpha5-precipitable material was subjected to immunoblotting with a polyclonal antiserum to beta1 subunit. Beta1-reactive bands were detected in the alpha5 precipitates both in control and transformed chondrocytes (not shown). Alpha5beta1 integrin has been shown to be a fibronectin receptor [66]. The alpha6 levels were significantly higher in RSV-transformed chondrocytes as compared to the levels displayed by differentiated CEC (Fig. 1E). Alpha6chain was detected as co-immunoprecipitated band after immunoprecipitation by a monoclonal anti-beta1-antibody both in CEC and in transformed chondrocytes (not shown).

The foregoing experiments all measure steady-state levels of integrins. To examine whether differences in the levels of the expression could be the result of enhanced rate of synthesis, we performed [ $^{35}$ S]-methionine metabolic labeling experiments. Lysates from metabolically labeled control CEC and NA 101 cells were immunoprecipitated with polyclonal antibodies raised against the cytoplasmic domains of chicken alpha2- and alpha3-chains and with a monoclonal antibody raised against the extracellular domain of chicken beta1 integrin subunit (Fig. 2A). A protein band with an apparent  $M_r$  below 116 kDa is immunoprecipitated by the anti-beta1-antibody from lysates of control chondrocytes. The immunoprecipitated proteins by the anti-alpha3-antibody were resolved into two bands, one in the  $M_r$  range of alpha3 subunit and the other in the  $M_r$  of the mature form of beta1 subunit. This last band and the one immunoprecipitated by the anti-beta1-antibody appear to be related by a precursor-product relationship, the mature form being observed as co-immunoprecipitated band after immunoprecipitation with the anti-alpha3-antibody (Fig. 2A). A protein with an apparent  $M_r$  of the mature form of the beta1 subunit was immunoprecipitated from RSV-transformed cells by the beta1 antibody together with a protein band of an apparent  $M_r$  of alpha3-chain. The identity of the alpha3-chain was confirmed by the immunoprecipitation with the specific anti-alpha3-antibody. Consistently with the results obtained by immunoblot, the rate of synthesis of alpha3-chain was higher in NA101 cells than in control chondrocytes. Similar results were obtained with freshly RSV-transformed chondrocytes (not shown).

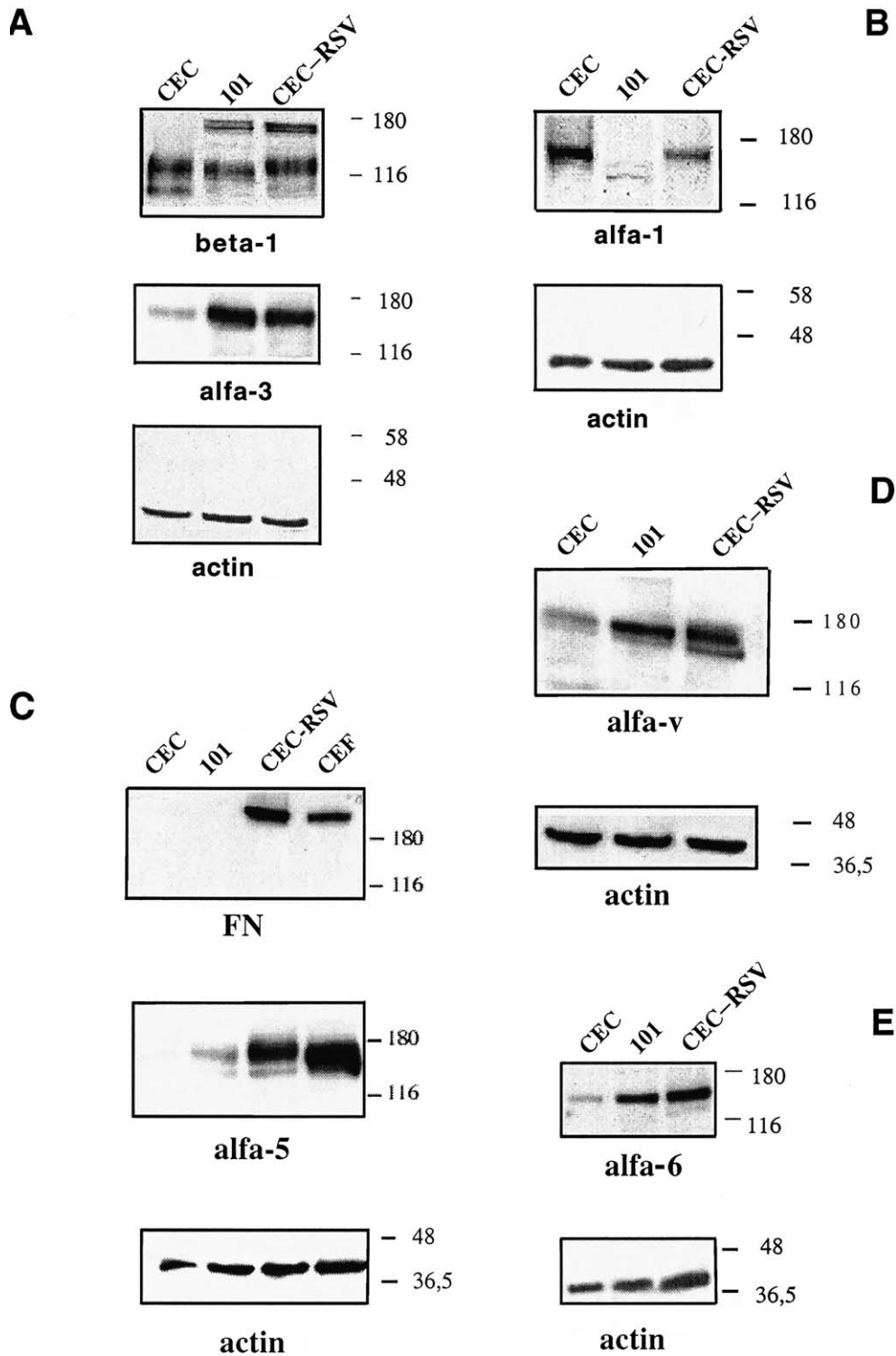


Fig. 1. Immunoblot analysis for the presence of different alpha- and beta-integrin subunits in normal and RSV-transformed chicken chondrocytes. Chicken epiphyseal chondrocytes (CEC), immortalized RSV-transformed chondrocyte, (NA101 cells), and freshly RSV-transformed chondrocytes (CEC-RSV). Twenty microgram of protein extracts were loaded per lane for each blot and electrophoresed on 7.5% SDS-PAGE under non-reducing conditions. Increased amounts of proteins (100 µg) were loaded on the gel depicted in panel B. Blots were incubated with polyclonal antibodies against the cytoplasmic domains of alpha3-, beta1-, alphav- and alpha5-subunits and monoclonal antibodies against the alpha6 subunit and fibronectin. In panel C, fibronectin detection was performed on a parallel gel run under reducing conditions. The higher molecular weight bands present in panel A were also present in the pre-immune serum. Actin detection by a monoclonal antibody was used to normalize protein loading. Immunoreactivity was visualized with HRP-conjugated anti-rabbit or anti-mouse antibodies. Numbers denote positions of  $M_r$  marker proteins in kiloDaltons.

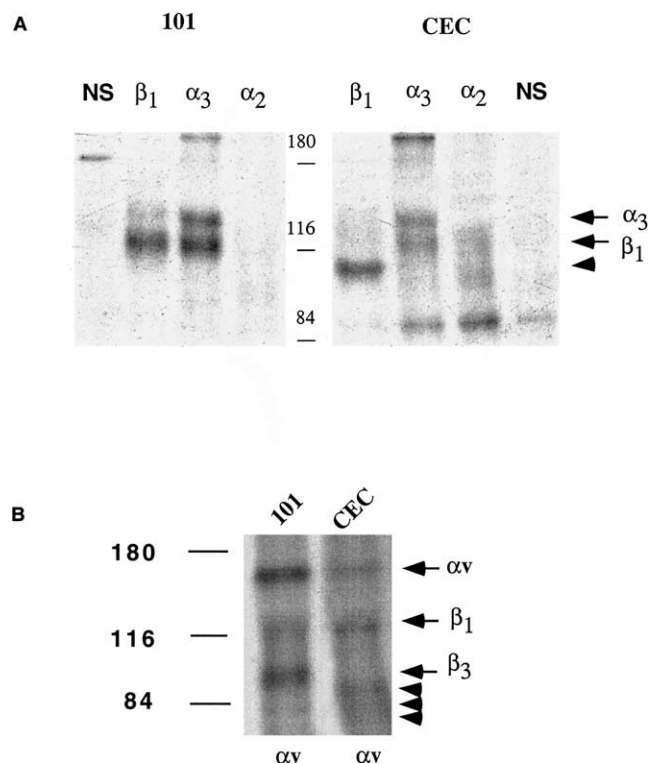


Fig. 2. (A) Rate of synthesis of beta1 integrins in normal and immortalized RSV-transformed chondrocytes. Immunoprecipitation of cell extracts from metabolically labeled cells: similar amounts of cpm were incubated with normal serum (NS), the monoclonal antibody against the extracellular domain of chicken beta1 integrin, the polyclonal antibodies against alpha3- or alpha2-chain. (B) Rate of synthesis of alphav-chain in normal and immortalized RSV-transformed chondrocytes. Immunoprecipitation of cell extracts from metabolically labeled cells. Similar amounts of cpm were incubated with the polyclonal antibody against the cytoplasmic domain of alphav-chain. Immunoprecipitated samples were resolved on 6% SDS-PAGE under non-reducing conditions. Numbers denote positions of  $M_r$  marker proteins in kiloDaltons.

Only barely detectable amounts of the alpha2-chain could be immunoprecipitated by the relevant antibody from control CEC (Fig. 2A). Likewise, barely detectable amounts of alpha1-chain could be immunoprecipitated from control and freshly transformed chondrocytes (not shown). Therefore it appears that normal and transformed chick chondrocytes fail to assemble significant amounts of collagen-binding alpha-beta1 containing alpha1- and alpha2-chains. Consistently with the results obtained by immunoblot, the rate of synthesis of alphav chain was higher in NA101 cells than in control chondrocytes (Fig. 2B). Similar results were obtained with freshly RSV-transformed chondrocytes (not shown). Distinct putative beta subunits were co-immunoprecipitated by the alphav antibody from metabolically labeled normal and v-Src-transformed chondrocytes (Fig. 2B). In addition to a protein with the same molecular weight as the beta1 subunits, protein bands of 82–94 kDa were also co-precipitated at the expected positions of the avian beta3- and beta5-chains [67]. The presence of the beta5 chain among the alphav-associated bands was confirmed by immunofluorescence with the help of a monoclonal alphavbeta5 specific antibody (not shown).

The identity of the beta3-chain detected as co-immunoprecipitated band, from metabolically labeled normal and transformed chondrocytes by the alphav antibody, was confirmed by immunoprecipitation experiments performed with the alphavbeta3 specific antibody, LM609 (not shown). alphav heterodimerizes with beta3 to a great extent in transformed chondrocytes (Fig. 2B).

Overall, the results indicate that normal and RSV-transformed chondrocytes express multiple alphav integrins as well as a number of beta1 integrins.

With the exception of alpha1- and alpha2-chains, the data are consistent with the enhanced biosynthesis of integrins in the transformed chondrocytes as compared with normal chondrocytes. Despite the higher levels of integrin expression, transformed chondrocytes display a refractile morphology and poorly attach to a number of substrates, including fibronectin, fibrinogen, vitronectin, collagens I and II, laminin (not shown).

#### 4. Discussion

Morphological transformation occurs independently of Src-induced nuclear changes [68] and most likely involves tyrosine phosphorylation of targets that control assembly and disassembly of the cellular actin and adhesion network [3–6]. Many v-Src targets are known to be associated with the cellular cytoskeletal network and focal adhesions, including the integrin beta1 subunit. Transformed cells have often defects in integrin function and localization [32–34].

This paper focuses on integrin expression in normal and RSV-transformed chick epiphyseal chondrocytes. Initially isolated as suspension cells, primary CEC soon attach to the plastic dish and assume the typical polygonal shape. With time in culture polygonal chondrocytes increase adhesion to the substrate, develop focal adhesions and stress fibers and dedifferentiate assuming a fibroblastic shape [27,44,45,63]. Differently v-Src-transformed chondrocytes show low adhesion activity, a refractile morphology and loss of focal adhesion and stress fibers [27,47,48]. Thus normal and transformed chondrocytes display different adhesion properties.

We wanted to determine whether the low adhesion activity of v-Src-transformed chondrocytes could be ascribed to a reduced integrin expression. v-Src-transformed chondrocytes were instead found to express increased levels of alpha3beta1, alpha6beta1 and alphavbeta3 integrins as compared with normal differentiated chondrocytes. Woods et al. [69] showed that selective and sustained activation of the Raf-MEK-ERK pathway in a variety of mouse fibroblasts and in mouse and human endothelial cells led to increased expression of a number of integrins, of which beta3- and alpha6-chains were the most prominent. However, despite the higher expression of integrins on the surface of NIH3T3 cells, Raf-transformed cells displayed decreased spreading and adhesion, with loss of focal adhesions and actin stress fibers. These data suggest that oncogene-induced alterations in integrin gene expression may participate in the changes in

cell adhesion that accompany the process of oncogenic transformation. Cells can vary their adhesive properties by altering the integrin expression or by modulating the integrin affinity state [35]. v-Src-induced alterations of the adhesive properties of chondrocytes might result from the regulation of the affinity state of beta1- and beta3-integrins. Recently, it has been shown that tyrosine phosphorylation in the NPXY motif of the cytoplasmic domain of beta1 subunit in v-Src-transformed cells contributes to the transformed phenotype by affecting integrin functions [24,70]. The NPXY motif is also required for tyrosine phosphorylation of beta3 subunit; Boettiger and colleagues [71] recently reported that phosphorylation of the cytoplasmic domain of beta3 negatively regulates alpha3beta3-fibronectin binding strength. Besides integrin phosphorylation, other regulatory mechanisms might be involved in the v-Src-induced alteration of the adhesive chondrocyte properties. It has been reported that sustained activation of cytoplasmic signaling pathways in transformed cells could be responsible for the suppression of integrin activation. Hughes et al. [72] found that the expression of activated variants of H-Ras and its kinase effector, Raf-1, suppress integrin activation and that this suppressive activity correlates with the activation of the ERK-MAP kinase pathway. They also showed that an activated form of Src shared with v-Src the ability to suppress integrin activation via a Ras-dependent MAP kinase pathway [72]. These data suggest that v-Src-induced transformation could produce a sustained activation of this integrin suppression pathway. These authors also found that the regulation of the integrin affinity state does not result from the phosphorylation of tyrosine residues on the beta3 cytoplasmic domain. In addition to the effects on global pattern of integrin expression, it is clear that the Raf-MEK-ERK pathway can influence the activation state of integrins by post-translational mechanisms. Recently, Ruoslahti and colleagues [73] reported that the R-Ras pathway also is, at least in part, responsible for the reduced adhesiveness of v-Src-transformed cells. Primary chick epiphyseal chondrocytes express alpha3-, alpha6-, alpha5-, beta1-, beta3-chains, with very low levels of alpha1-, alpha2- and alpha5-chains. Two bands by the anti-beta1-integrin antibody were observed both in immunoblot and immunoprecipitation experiments performed on normal chondrocytes growing in monolayer. These two bands could be related by a precursor-product relationship. By pulse-chase experiments, we observed a slow rate of maturation of the beta1 subunit in control chondrocytes as compared with the previously reported data ([65], the results are not shown and have been made available to the reviewers). Chick epiphyseal chondrocytes can grow in suspension or in monolayer and these two cell populations convert one into the other at least in young cultures. A slow rate of maturation of the beta1 subunit could affect chondrocyte integrin functions and give rise to cells able to float in the medium.

It appears that normal and transformed CEC fail to assemble significant amounts of alpha1beta1 and alpha2beta1 integrin, two of the best-known collagen receptors, and ex-

press instead high levels of alpha3beta1 integrin. In other cell types, alpha3beta1 serves as an alternative or secondary receptor for collagen, laminin or fibronectin [74]. So far, alpha1-, alpha2- and alpha10beta1 have been found to mediate the binding of chondrocytes to collagen II [75–78], with alpha2-beta1 involved in the binding of chondroadherin as well [79]. The lack of significant amounts of alpha1- and alpha2-chains in differentiated chondrocytes, may indicate either that on these cells other receptors, besides integrins, may mediate cell-collagen interactions [80], or that another alpha chain is involved in the interaction with collagen II. The isolation of alpha10beta1 integrin by affinity chromatography on type II collagen suggests that this integrin may be a key receptor for this collagen [77]. The expression of the alpha10 chain in CEC has not been detected because of lack of cross-reactivity of the antibody that these authors have kindly provided to us. The lack of significant amounts of alpha2 subunit in CEC is consistent with previous findings, showing that alpha2 could not be identified in human fetal epiphyseal cartilage [75] and in adult articular chondrocytes [81]. Controversial reports concerning alpha1 expression in articular chondrocytes exist in literature with some reports showing alpha1 and its affinity for collagen II and VI [78] and others reports showing weak or no expression of alpha1 in adult articular cartilage and in human fetal epiphyseal cartilage [75,81].

As to the alpha3beta1 expression by chondrocytes, it has been suggested a strict correlation between the collagen type synthesized and the appropriate receptor presented by chondrocytes in organoid cultures from mouse limb buds. In fact, Shakibaei et al. [82] reported that the change in collagen production from type I to type II during chondrogenesis was accompanied by a change in integrin expression from alpha1- to alpha3-chain. Conversely, dedifferentiation of chondrocytes in aging cartilage was accompanied by the occurrence of collagen type I and alpha1 integrin subunit. Differently, it has been reported that the alpha3beta1 on two chondrosarcoma cell lines appears not to interact with collagen II [76,83]. Here, the change in the collagen production from type II to type I collagen in freshly transformed chondrocytes is associated with the increased expression of the alpha3 chain and the reduced expression of alpha1 chain. The alpha3 integrin chain is still highly expressed by established NA101 cells, in the absence of significant production of cartilage collagens and collagen type I.

Alpha6 chain is detected as co-immunoprecipitated band after immunoprecipitation by anti-beta1 antibody both in normal and in transformed chondrocytes. Alpha6beta1 heterodimer functions as a laminin receptor. This is consistent with previous findings by Durr et al. [75], who showed the presence of alpha6 on human fetal chondrocytes from epiphyseal cartilage and the block of chondrocyte attachment on laminin by a monoclonal anti-beta1-antibody. RSV-transformed chondrocytes were found to accumulate higher levels of alpha6beta1 as compared with normal differentiated chondrocytes. Both the heterodimers, alpha3beta1 and

alpha6beta1 display higher expression in RSV-transformed chondrocytes. High expression of the integrin subunits alpha6 and alpha3 is associated with transformation and tumor progression (for reviews see Refs. [33,34]). Early findings described a decrease in the expression of the fibronectin receptor, alpha5beta1 integrin, in transformed cells [84]. We observed that the low expression of alpha5 subunit in normal chondrocytes increases in freshly transformed cells; low levels of this subunit were found in the immortalized transformed cells as compared with freshly transformed cells. Alpha5beta1 is the high affinity receptor specific for fibronectin and the relative expression of alpha5 subunit correlates with the expression of fibronectin in normal and transformed chondrocytes, in that the extents of alpha5 and fibronectin expression are closely linked. Despite repeated subcloning, fibronectin expression by the immortalized NA101 cells decreases upon subculture. Transcriptional repression of fibronectin gene by v-Src has been demonstrated (reviewed in Ref. [64]).

A polyclonal anti-alphav-antibody recognizes specific integrin heterodimers in normal and RSV-transformed chondrocytes. Distinct putative beta chains co-immunoprecipitated with the alphav chain. The presence of alphavbeta1, alphavbeta3 and alphavbeta5 heterodimers in normal and transformed chondrocytes were found. The expression of the beta3 chain in chondrocytes is consistent with the results by von der Mark and colleagues [75], who showed the presence of the vitronectin receptor both on freshly isolated chondrocytes from human epiphyseal cartilage and in situ. Increased levels of alphav subunit were found in transformed chondrocytes. Alphav subunit in transformed cells heterodimerizes to a great extent with the beta3 subunit. Although alphavbeta3 integrin is known as the receptor for vitronectin it binds to other RGD-containing ECM proteins, such as fibrinogen, osteopontin, bone sialoprotein, and, to some extent, fibronectin and a cryptic RGD-site in denatured collagen. Expression of this integrin enables a given cell to adhere to, migrate on, or respond to almost any matrix protein it may encounter. In spite of the high expression of this heterodimer in transformed chondrocytes, these cells display low affinity for a number of substrates including fibrinogen, vitronectin and fibronectin as determined by cell adhesion assays. Thus it appears that the activation of the v-Src signaling pathway in chondrocytes can have profound effects on the expression and the activity of these key cell adhesion molecules.

## 5. Conclusions

Normal and v-Src-transformed epiphyseal chondrocytes display different adhesion properties. While differentiated CEC become increasingly adherent with time in culture, transformed chondrocytes display low spreading, loss of focal adhesions and stress fibers and low adhesion activity to several ECM constituents.

The objective of this study was to determine how the expression of the integrin receptors was modulated after v-Src-induced transformation of cultured chondrocytes. We found that alpha3beta1, alpha6beta1 and alphavbeta3 integrins are expressed in greater amounts in RSV-transformed chondrocytes as compared with the levels expressed by control CEC. These data indicate that the effects of v-Src on cell morphology, intracellular architecture and signaling run counter to the simple expectation that cell-ECM attachment might be increased in these cells and lend support to the idea that v-Src can affect the integrin adhesive functions.

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