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Up-regulation of the integrin $\alpha 1/\beta 1$ in human neuroblastoma cells differentiated by retinoic acid: correlation with increased neurite outgrowth response to laminin

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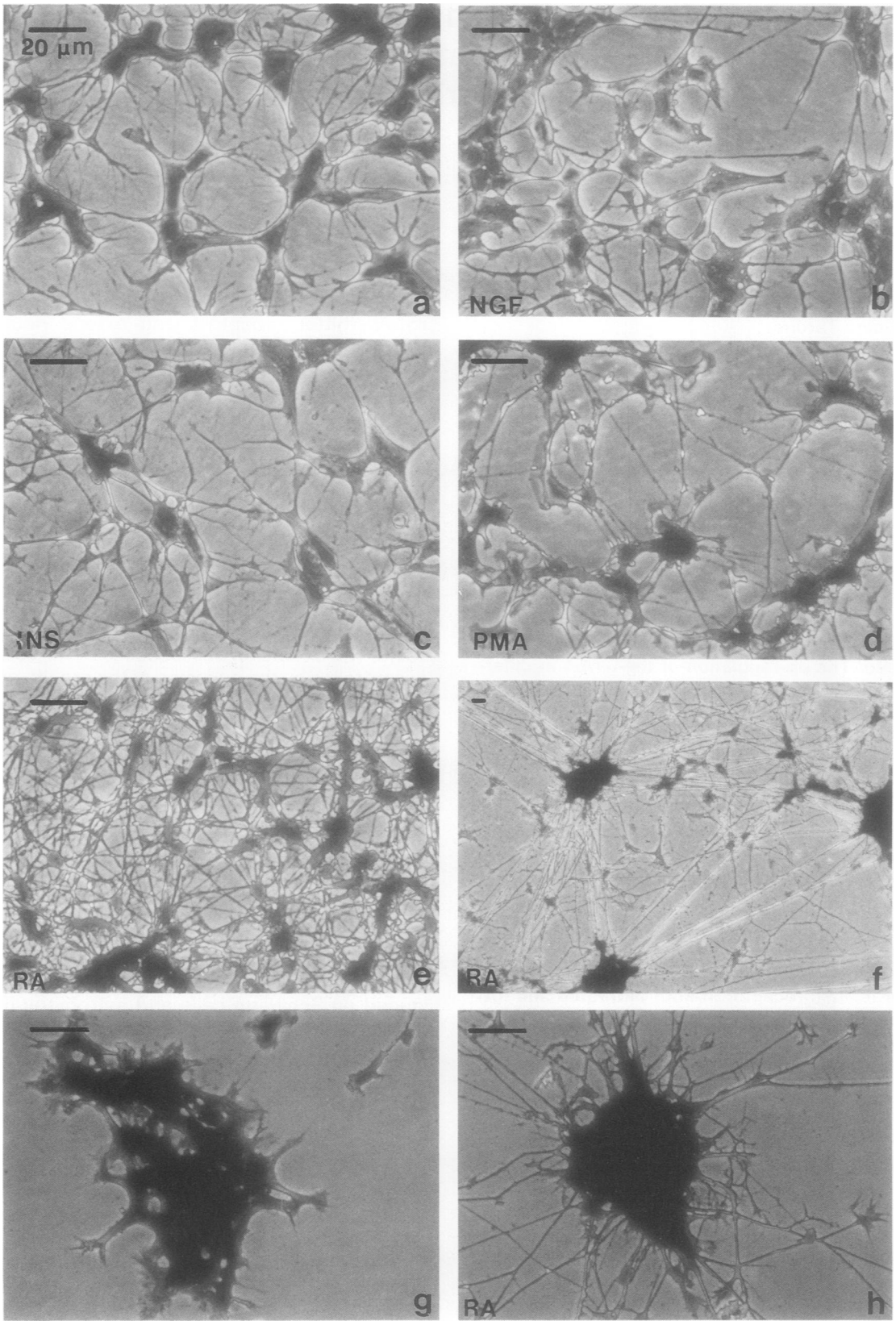
Retinoic acid (RA) is known to induce differentiation of neuroblastoma cells in vitro. Here we show that treatment of two human neuroblastoma cell lines, SY5Y and IMR32, with RA resulted in a fivefold increase of the integrin $\alpha 1/\beta 1$ expression. The effect was selective because expression of the $\alpha 3/\beta 1$ integrin, also present in these cells, was not increased. The up-regulation of the $\alpha 1/\beta 1$ in differentiated SY5Y cells correlated with increased neurite response to laminin. In fact, RA-treated SY5Y cells elongated neurites on laminin-coated substratum more efficiently compared with untreated cells or cells treated with nerve growth factor, insulin, or phorbol 12-myristate 13-acetate. These three agents induced partial morphological differentiation but did not increase $\alpha 1$ integrin expression. Neurite extension in RA-treated cells was more efficient on laminin than on fibronectin or collagen type I and was inhibited with $\beta 1$ integrin antibodies on all three substrates. Affinity chromatography experiments showed that $\alpha 1/\beta 1$ is the major laminin receptor in both untreated and RA-treated SY5Y cells. These data show that RA, a naturally occurring morphogen implicated in embryonic development, can selectively regulate the expression of integrin complexes in neuronal cells and suggest an important role of the $\alpha 1/\beta 1$ laminin receptor in the morphological differentiation of nerve cells.

Introduction

The SY5Y and the IMR32 human neuroblastoma cell lines are a useful model system to study neuronal differentiation. These cells, in fact, synthesize multiple neurotransmitters and neurotransmitter receptors and extend neurites (Biedler *et al.,* 1978; Pahlman *et al.,* 1981; Recio-Pinto and Ishii, 1984; Clementi *et al.,* 1986; Adem *et al.,* 1987) when treated with various agents, including nerve growth factor (NGF) (Perez-Polo *et al.,* 1979; Sonnenfeld and Ishii, 1982), insulin (Recio-Pinto and Ishii, 1984), dibutyryl-cyclic AMP (De Laat and van der Saag, 1982), bromo-deoxyuridine (Prasad, 1975), phorbol 12-myristate 13-acetate (PMA) (Pahlman *et al.,* 1981; Spinelli *et al.,* 1982), and retinoic acid (RA) (Pahlman *et al.,* 1984).

Using these cell lines we investigated the role of integrins in neurite extension on matrix proteins. The extension of processes by nerve cells is controlled by the adhesion of the growth cone, a specialized structure of the growing process, with other cells and with extracellular matrix (Reichardt *et al.,* 1989; Sanes, 1989; Lander, 1990). Several cell–cell adhesion molecules, including N-CAM, Ng-CAM, and N-Cadherin, play an important role in supporting neurite extension by mediating neuron–neuron, neuron–glia, or neuron–muscle interactions (Bixby *et al.,* 1987, 1988; Neugebauer *et al.,* 1988; Tomaselli *et al.,* 1988). Among the matrix components, laminin has an important role as it promotes neurite outgrowth and extension of several nerve cell types (Sanes, 1989). Laminin is produced by Schwann cells and astrocytes (Liesi *et al.,* 1983; Bunge *et al.,* 1989) and is a basal membrane component in the peripheral nerves (Bunge *et al.,* 1989) and in selected regions of the central nervous system (Liesi, 1985). Nerve cells recognize laminin via cell-surface receptors of the integrin family (Reichardt *et al.,* 1989). Two major laminin-binding integrins, $\alpha 1/\beta 1$ and $\alpha 3/\beta 1$, have been identified in neuronal cells in culture and support neurite extension (Rossino *et al.,* 1990; Tomaselli *et al.,* 1990).

In this paper we show that differentiation of SY5Y and IMR32 neuroblastoma with RA induced a selective up-regulation of the integrin


20 µm
a
NGF
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RA
e
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$\alpha 1/\beta 1$, a laminin and collagen receptor. Increased expression of this receptor potentiates the neurite extension on laminin indicating that this may represent a mechanism relevant for morphological differentiation of nerve cells.

Results

Morphological differentiation in human neuroblastoma cells with different agents

Human neuroblastoma cell lines SY5Y and IMR32 were shown to undergo neuronal differentiation when exposed to several stimuli including NGF, insulin, PMA, and RA (Perez-Polo *et al.,* 1979; Pahlman *et al.,* 1981, 1984; Recio-Pinto and Ishii, 1984). To evaluate the effect of these agents on the morphological differentiation, neuroblastoma cells were grown for different length of time with the various factors on uncoated dishes in medium with 10% serum. After treatment for 7 d with NGF, insulin, or PMA, SY5Y cells displayed neurites longer and more numerous than untreated cells (Figure 1, a–d). Increasing the concentration of these agents or prolonging the treatment for 25–30 d did not further increase the degree of morphological differentiation reached after 7 d treatment in standard condition (see Materials and methods). Treatment with RA resulted in more pronounced morphological differentiation of SY5Y cells. As shown in Figure 1e, RA-treated cells became smaller and round and extended a dense and intricate network of neuritic processes. The morphological differentiation was initially appreciated after 3 d of treatment with 10^{-5}M RA and proceeded during the next 25–30 d until the cells aggregated in pseudoganglion structures connected by long fascicles of neurites (Figure 1f). Lower concentrations of RA (10^{-6} and 10^{-7}M) were also effective in inducing morphological differentiation but in these cases a longer treatment was required. IMR32 cells also showed a dramatic response to RA (Figure 1). These cells tend to form clumps and are poorly adherent to the culture dish when grown in standard conditions (Figure 1g). After 7 d of RA treatment, cell clumps were more adherent to the substratum and showed extended processes with typical growth cones (Figure 1h). As for SY5Y, IMR32 cells did show a much less pronounced degree of morphological differentiation when treated with either NGF, insulin, or PMA.

RA induces increased expression of the $\alpha 1$ integrin

To investigate whether there was a relationship between morphological differentiation of these cells and integrin expression, SY5Y cells were labeled with ^{35}S-methionine, and integrin complexes were immunoprecipitated with subunit-specific antibodies. As shown in Figure 2 (lanes a–c), these cells express predominantly two integrin complexes corresponding to $\alpha 1/\beta 1$ and $\alpha 3/\beta 1$. $\alpha 2$, $\alpha 6$, and αV subunits were barely detectable, whereas $\alpha 4$ and $\alpha 5$ were absent. $\alpha 1$ and $\alpha 3$ subunits also represent the two major integrins in IMR32 neuroblastoma cells (not shown) suggesting that this pattern is somehow typical of neuronal cells.

Analysis of SY5Y cells treated with different agents showed that NGF, insulin, and PMA did not cause significant alteration in the integrin pattern observed in untreated cells (Figure 2, lanes d–g). On the other hand, RA treatment induced a selective increase in $\alpha 1$ integrin (Figure 2, lanes h and j). The expression of $\alpha 2$, $\alpha 6$, and αV (not shown) was not increased after treatment with RA, whereas the expression of $\alpha 3$ was slightly decreased as compared with untreated cells (Figure 2, lanes d, h, i, and j).

$\alpha 1$ levels in RA-treated cells were five times higher than in control cells as measured by metabolic labeling, and the $\alpha 1/\beta 1$ complex was expressed at the cell surface as shown by surface radioiodination (Figure 2, lanes m and n). Specific up-regulation of $\alpha 1$ integrin was also observed in IMR32 neuroblastoma cells treated with RA (Figure 2, lane l). Analysis of the kinetics of induction indicated that increased levels of $\alpha 1/\beta 1$ integrin were detectable after 48 h of treatment with 10^{-5}M RA. To investigate the mechanisms leading to up-regulation of the $\alpha 1/\beta 1$ integrin complex, the synthesis of both $\alpha 1$ and $\beta 1$ subunits was analyzed by pulse-chase experiments. As shown in Figure 3, incorporation of methionine in $\alpha 1$, but not in $\alpha 3$ and $\beta 1$,

Figure 1. **Morphology of SY5Y and IMR32 human neuroblastoma cells treated with different agents.** Cells, grown on uncoated dishes with the appropriate factor for the indicated period of time in 10% serum, were fixed, stained, and photographed. (a) Untreated SY5Y cells. SY5Y cells exposed to (b) 100 ng/ml NGF for 7 d, (c) 1 μg/ml insulin for 7 d, (d) 1.6×10^{-8} M PMA for 7 d, (e) 1×10^{-5} M RA for 7 d, (f) 1×10^{-5} M RA for 25 d. (g) Untreated IMR32 cells, (h) IMR32 cells treated with 1×10^{-5} M RA for 7 d. All pictures are at the same magnification (500×) except for (f) (125×). Bars = 20 μm.

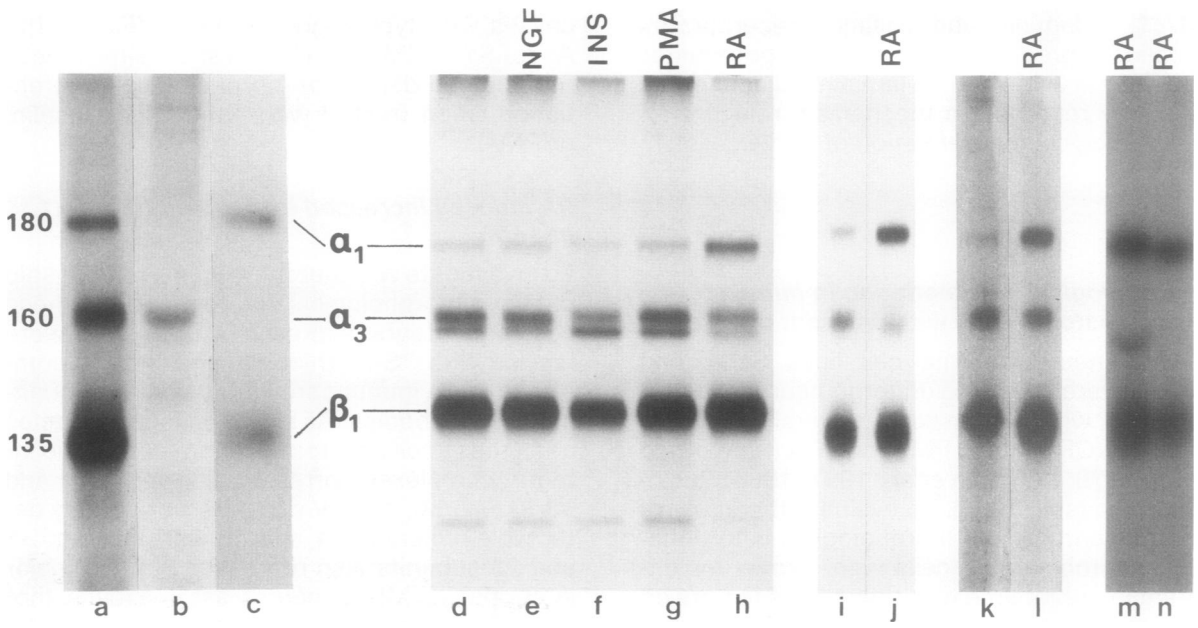

Figure 2. **Integrin expression in human neuroblastoma cells.** (lanes a–c) SY5Y cells were labeled for 15 h with ^{35}S-methionine and detergent extracts were immunoprecipitated with antibodies to FN-R ($\alpha5/\beta1$) (a), $\alpha3$ (b), and $\alpha1$ (c). The FN-R serum recognizes the $\beta1$ subunit because these cells do not express $\alpha5$. (lanes d–h) SY5Y cells were grown for 7 d in standard conditions (d) or in the presence of 100 ng/ml NGF (e), 1 μg/ml Insulin (f), 1.6×10^{-8} M PMA (g), 1×10^{-5} M RA (h). Cells were labeled for 6 h with ^{35}S-methionine, extracted with detergent, and immunoprecipitated with antibodies to FN-R. Note that $\alpha1$ subunit is specifically increased in RA-treated cells (h). The lower band of the doublet in the $\alpha3$ region represents an immature form of the $\alpha3$ subunit detectable in cells labeled for 6 h (d–h) but not in those labeled for 15 h (lanes a–c and i–l). (lanes i–l) Cells were labeled with ^{35}S-methionine for 15 h and immunoprecipitated with FN-R antibodies: untreated SY5Y cells (i); SY5Y cells treated with 1×10^{-5} M RA for 7 d (j); untreated IMR32 cells (k); IMR32 cells treated with 1×10^{-5} M RA for 7 d (l). (lanes m–n) SY5Y cells treated with 1×10^{-5} M RA for 7 d were surface-labeled with ^{125}I and lactoperoxidase. Detergent extracts were immunoprecipitated with antibodies to $\beta1$ (m) and $\alpha1$ (n) subunits. The numbers on the left indicate the relative molecular mass of $\alpha1$, $\alpha3$, and $\beta1$ expressed in kDa.

was increased after RA treatment. Densitometric analysis indicated a 4.5-fold increase in $\alpha1$. Partial degradation of $\alpha1/\beta1$ occurred only after 6 h of chase in both untreated and treated cells at comparable rate. Thus, the increased expression of the $\alpha1/\beta1$ complex is largely due to a selective stimulation of $\alpha1$ synthesis.

The lack of stimulation of $\beta1$ synthesis is somehow puzzling because increasing amounts of $\alpha1$ will require proportionally more $\beta1$ to complex with. The results of the pulse-chase experiment provided a possible explanation for this apparent discrepancy and also revealed some interesting features of the mechanisms of α/β association. The formation of the α/β complexes occurred within the first 30 min of chase (Figure 3, lane 2 in panels 1 and 3) as indicated by the ability of $\beta1$-specific antibodies to coprecipitate $\alpha1$ and $\alpha3$ subunits. However, $\alpha1$-specific antibodies did not coprecipitate labeled $\beta1$ during the first 6 h of chase (Figure 3, lanes 1–5 in panels 2 and 4). Coprecipitation of labeled $\beta1$ was appreciable only after 15 h of chase (Figure 3, lane 6 in panel 2 and 4). Thus, during the first 6 h of chase the complex consists of labeled $\alpha1$/unlabeled $\beta1$. These results can be explained by the presence of a large intracellular pool of $\beta1$ subunit that greatly dilutes the molecules labeled during the pulse. This preexisting pool will also function as a store providing the $\beta1$ molecules required to match the increasing amount of $\alpha1$.

RA-treated cells show increased neurite response to laminin

To analyze whether the altered integrin expression in RA-treated cells was relevant to the organization of neuritic processes, we evaluated neurite extension in response to specific matrix proteins.

We first analyzed the neurite elongation on laminin because the role of this molecule in supporting neurite outgrowth is well docu-

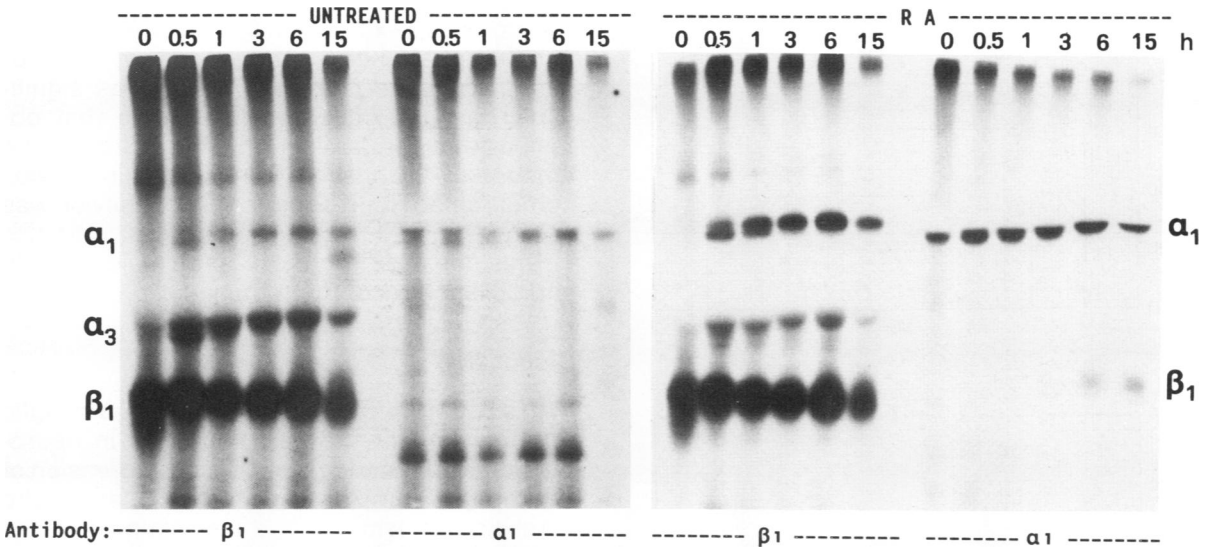

Figure 3. **Analysis of $\beta 1$ and $\alpha 1$ integrin synthesis by pulse-chase experiments.** SY5Y cells untreated (left) or treated with 1×10^{-5} M RA for 7 d (right) were pulsed for 15 min with ^{35}S-methionine and chased for the indicated lengths of time (0, 0.5, 1, 3, 6, 15 h). At the end of the chase time, cells were detergent-extracted and TCA precipitable counts were determined. Equal amounts of counts were taken from each sample and subjected to immunoprecipitation with polyclonal antibodies to the COOH-terminal peptide of $\beta 1$ and with monoclonal antibody (TS2/7) to $\alpha 1$. The position of $\alpha 1$, $\alpha 3$, and $\beta 1$ subunits are shown.

mented (Martin and Timpl, 1987; Sanes, 1989). Cells were treated with the various agents for 7 d in culture, detached with ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), and plated for 2 h in serum-free medium on laminin-coated dishes. These conditions differ from those used in Figure 1 because in that case exogenous matrix proteins were not added and cells were allowed to produce their own neurite-promoting factors. In fact, cells were grown on uncoated dishes for 7 d in the presence of 10% serum. As shown in Figure 4, untreated SY5Y cells responded to some extent to laminin; about 40% of the cells extended processes and the mean length was 20 μm. Cells treated with NGF, insulin, or PMA did not differ significantly in their response from untreated cells (Figure 4). On the other hand, RA treatment effectively increased the number of cells with processes (94%) as well as the mean length of the neurites (36.5 μm). The increased ability of RA-treated cells to elongate neurites on laminin is also demonstrated by the fact that these cells displayed a high number of neurites (40%) longer than 45 μm. In untreated cells and in cells treated with NGF, insulin, or PMA, the percentage of neurites longer than 45 μm ranged from 0% (insulin) to 15% (PMA) (Figure 5). In conclusion, treatment with all four agents stimulated various degrees of neurite extension in cells grown for 7 d on uncoated dishes in serum-containing medium (see Figure 1). However, when these cells were detached and plated on coated dishes, only RA-treated cells showed an increased response to laminin.

We then compared the response of untreated and RA-treated cells with different matrix proteins. RA-treated cells extended neurites more efficiently on all three substrates (Figure 6). However, the stimulation was more pronounced in cells plated on laminin compared with those on fibronectin or type I collagen. As indicated in Figure 6, in fact, the number of cells with neurites increased threefold on laminin (from 27% to 92%), whereas it increased twofold in cells plated on fibronectin (from 28% to 54%) or on type I collagen (from 16% to 35%).

Adhesion and neurite extension of both untreated and RA-treated cells on the three matrix proteins is mediated by integrin complexes as indicated by the fact that antibodies to the integrin $\beta 1$ subunit detached cells from all three substrates (Table 1).

Identification of the laminin receptor

Affinity chromatography experiments were performed to identify the integrin complex that mediates laminin adhesion in SY5Y cells. Membrane proteins were labeled by surface radio-

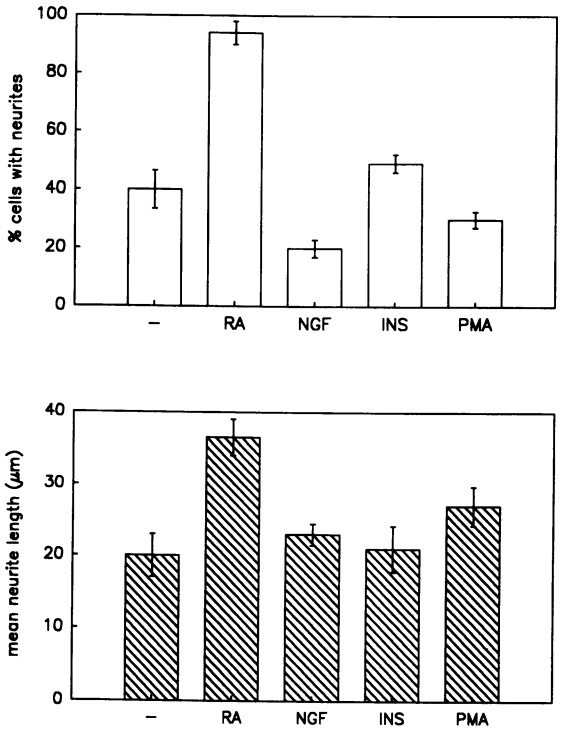

***Figure 4.* Neurite extension on laminin by SY5Y cells treated with different agents.** Cells treated with different agents as described in Materials and methods were harvested with EGTA and plated in RPMI medium without serum for 2 h on multiwells plates coated with 10 μg/ml of laminin. After fixation and staining with crystal violet, five randomly selected fields were photographed for each sample. Process were measured in 150 cells per sample and only those longer than 15 μm were scored.

iodination and cell extracts were chromatographed through a laminin column. As shown in Figure 7, the $\alpha 1/\beta 1$ complex bound to laminin and it was specifically eluted with EDTA known to interfere with binding of integrins to their ligands (Pytela *et al.*, 1987). Further elution with a carbonate buffer at pH 11 did not release labeled material. Because it has been reported that $\alpha 1/\beta 1$ integrin from human melanoma cells binds to collagen (Kramer and Marks, 1989), we tested whether this was the case also in neuroblastoma cells. As shown in Figure 7, the $\alpha 1/\beta 1$ complex also bound to a collagen type I column and was specifically eluted with EDTA. The proteins eluted from both the laminin and collagen columns were specifically immunoprecipitated with antibodies to $\beta 1$ and to $\alpha 1$ subunits confirming the identity of the receptor as $\alpha 1/\beta 1$ integrin. On both columns the amount of bound receptor was higher in RA-treated cells compared with untreated cells in agreement with the increased expression of the $\alpha 1/\beta 1$ complex in differentiated cells. The amount of receptor eluted from the collagen column was significantly and reproducibly higher than that obtained from the laminin column, although the amount of ligands on the two columns was comparable. The reason for this behavior was not investigated.

Thus, the $\alpha 1/\beta 1$ complex is the major laminin and collagen receptor in these cells.

Discussion

In this paper we show that RA induces a specific up-regulation of the $\alpha 1/\beta 1$ integrin in neuroblastoma cells and that increased expression of this integrin complex leads to increased neurite extension on laminin. It is interesting that the ability of cells treated with NGF, insulin, or PMA to organize neurite when plated on laminin-coated dishes is not significantly different from that of untreated cells. None of these agents enhanced the expression of the $\alpha 1/\beta 1$ integrin.

The effect of RA on $\alpha 1/\beta 1$ expression is specific because the expression of $\alpha 3/\beta 1$ is not increased in the same conditions. This behavior is reminiscent of what we observed previously in the rat neuronal cell line PC12 (Rossino *et al.*, 1990). In this system we have demonstrated that NGF induces a specific up-regulation of a laminin-binding integrin that is the rat equivalent of the human $\alpha 1/\beta 1$ complex (Tomaselli *et al.*, 1990). As for RA-treated SY5Y cells, increased expression of the $\alpha 1/\beta 1$ integrin in NGF-treated PC12 cells correlated to increased neurite response to laminin. Thus, up-regulation of the $\alpha 1/\beta 1$ is common to different neuronal cell lines.

Based on our previous data on PC12 cells and on the fact that both PC12 and SY5Y cells express high-affinity NGF receptors (Schechter and Bothwell, 1981; Sonnenfeld and Ishii, 1982), we expected NGF to induce increased expression of the $\alpha 1/\beta 1$ integrin in the human neuroblastoma SY5Y. As shown in Figure 2e, this was not the case and demonstrates that the two cell lines respond differently to NGF. This is also indicated by the fact that NGF arrested cell growth in PC12 cells, but it did not in SY5Y and induced extensive morphological differentiation only in PC12 cells.

Integrin $\alpha 1/\beta 1$ and $\alpha 3/\beta 1$ represent the major integrin complexes of the two human neuroblastoma cell lines SY5Y and IMR32 (this paper) and of the rat neuronal cell line PC12 (Rossino *et al.*, 1990; Tomaselli *et al.*, 1990). Several data indicate that both these integrin complexes

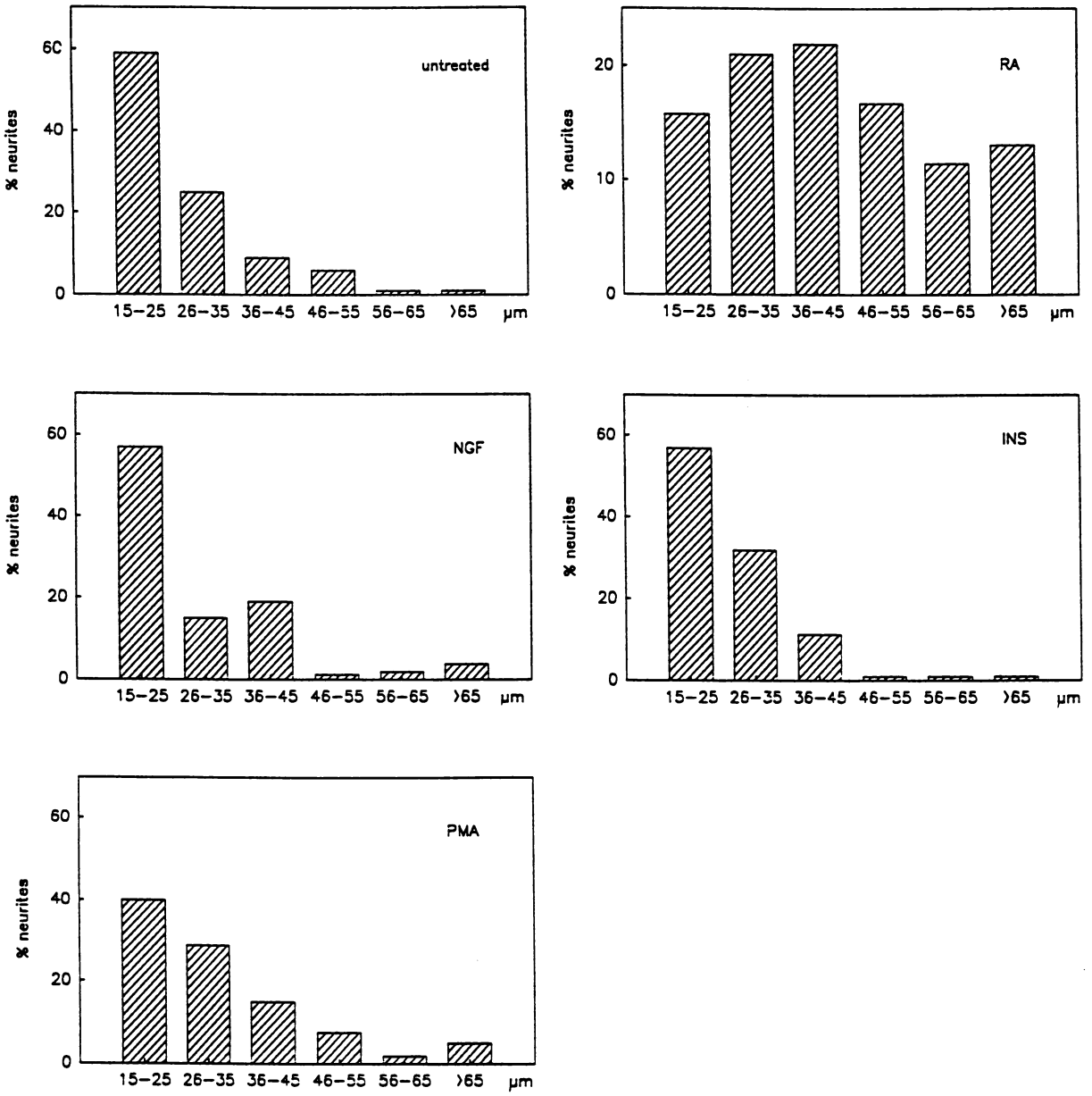

Figure 5. **Size distribution of neurites in SY5Y cells treated with different agents and plated on laminin-coated dishes.** The data are derived from the experiment described in Figure 4. Each bar represent the percentage of neurites (ordinate) falling into a given range of length expressed in μm (abscissa). Note that the majority of neurites in untreated cells or in cells treated with NGF, insulin, or PMA have length ranging from 15 to 45 μm, whereas RA-treated cells have a high percentage of neurites longer than 45 μm.

mediate neurite extension on laminin and collagen but they have distinct functional properties. In fact, 1) $\alpha 3/\beta 1$ integrin seems to have a weaker interaction with its ligands compared with $\alpha 1/\beta 1$ as indicated by antibody inhibition studies (Tomaselli *et al.*, 1990). A complete inhibition of neurite extension requires a combination of $\alpha 1$ and $\alpha 3$ antibodies. However, $\alpha 3$ antibodies alone are ineffective, whereas antibodies to $\alpha 1$ partially inhibit neurite extension (Turner *et al.*, 1989; Tomaselli *et al.*, 1990). 2) The $\alpha 1/\beta 1$ integrin binds both laminin and collagens, as shown by affinity chromatography experiments (Ignatius and Reichardt, 1988; Kramer and Marks, 1989; Forsberg *et al.*, 1990, Rossino *et al.*, 1990; this paper). The $\alpha 3/\beta 1$

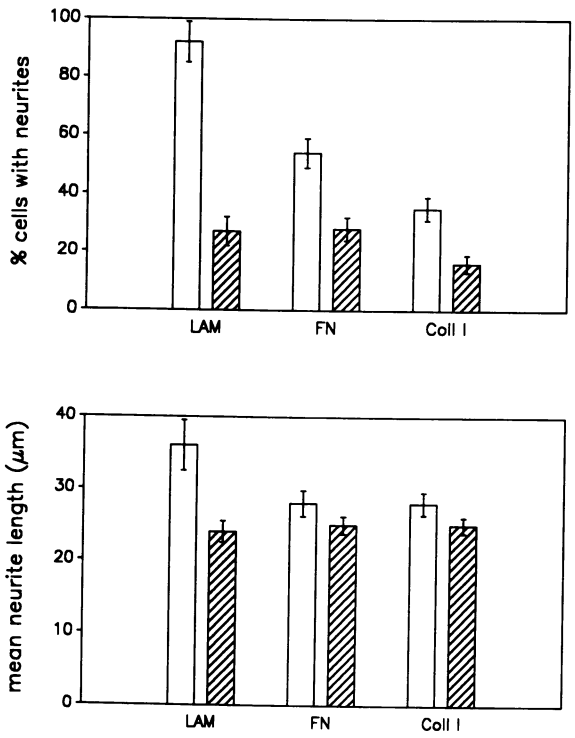

Figure 6. **Neurite extension by untreated and RA-treated SY5Y cells on different matrix proteins.** Cells untreated (□) or treated with 1×10^{-5} M RA for 7 d (▨) were detached with EGTA and plated in serum-free medium for 2 h on dishes coated with 10 μg/ml of laminin (LAM), 10 μg/ml of fibronectin (FN), or 10 μg/ml of collagen type I (Coll I). After fixation and staining with crystal violet, five randomly selected fields were photographed for each sample. Processes were measured in 150 cells per sample and only those longer than 15 μm were scored.

complex shows weak (Elices *et al.*, 1991) or no binding (this paper) to the A,B1,B2 mouse laminin and to collagen type I. Thus, the two integrins differ in the affinity for these ligands. Other authors (Gehlsen *et al.*, 1988) reported effective binding of the α3/β1 complex to human placenta laminin known to consist of a mixture of isoforms (Leivo and Engvall, 1988). This suggests that α3/β1 may bind preferentially to a specific laminin isoform distinct from the A,B1,B2 form isolated from the mouse tumor and used in our studies. 3) α1/β1 binds to the P1 (Rossino *et al.*, 1990) or E1 (Hall *et al.*, 1990) region corresponding to the central domain of the cross-shaped laminin molecule, whereas α3/β1 (Gehlsen *et al.*, 1989; Tomaselli *et al.*, 1990) recognizes the E8 domain corresponding to the lower region of the long arm of the cross (Martin and Timpl, 1987). Both P1/E1 and E8 domains promote neurite outgrowth and extension *in vitro* (Edgar *et al.*, 1984; Rossino *et al.*, 1990; Tomaselli *et al.*, 1990). 4) As discussed above, both in rat pheochromocytoma PC12 cells and in human neuroblastoma cell lines the α1/β1 integrin, but not the α3/β1, is up-regulated during neuronal differentiation.

Analysis of the mechanisms leading to α1/β1 up-regulation in RA-treated cells indicated that stimulation of α1 synthesis accounted for most, if not all, of the increased expression. Interestingly, the synthesis of the companion β1 subunit was not increased and the β1 molecules required to form the complex came from a preexisting intracellular pool. Other authors have also noted that β1 accumulates in intracellular stores in excess with respect to α subunits (Heino *et al.*, 1989). This may indeed represent an interesting and convenient mechanism allowing the cells to modify the expression of a given integrin complex by simply altering the level of the corresponding α subunit. If β would be present within the cells in amounts just sufficient to associate with the available α subunits, more complex mechanisms of coordinate control of α and β will be required to regulate integrin expression.

The α1/β1 complex in SY5Y cells bound to both laminin and collagen type I. This double specificity has been reported also in other cellular systems (Clyman *et al.*, 1990). It is of interest that neurite elongation by RA-treated cells expressing high levels of α1/β1 is more pronounced on laminin than on collagen type I. The reason of this behavior remains unclear.

Our data indicate that NGF, insulin, and PMA, although capable to induce neuronal differen-

Table 1. Detachment of SY5Y cells with antibodies to β1 integrin

Treatment	Substratum	Adherent cells (%)[a]
—	Laminin	10
RA	Laminin	15
RA	Fibronectin	9
RA	Collagen I	12
RA	Polylysine	98

[a] Cells, treated for 7 d with RA or untreated control cells were harvested with EGTA and allowed to adhere for 15 h on microtiter wells coated with the indicated protein (Substratum) in medium without serum. Cells were then incubated for 2 h at 37°C with serial dilutions of the antiserum to human fibronectin receptor or of the preimmune serum. After washing, adherent cells were fixed, stained, and counted. The values reported are measured at 1/400 antiserum dilution. The percentage is referred to adherent cells in preimmune serum.

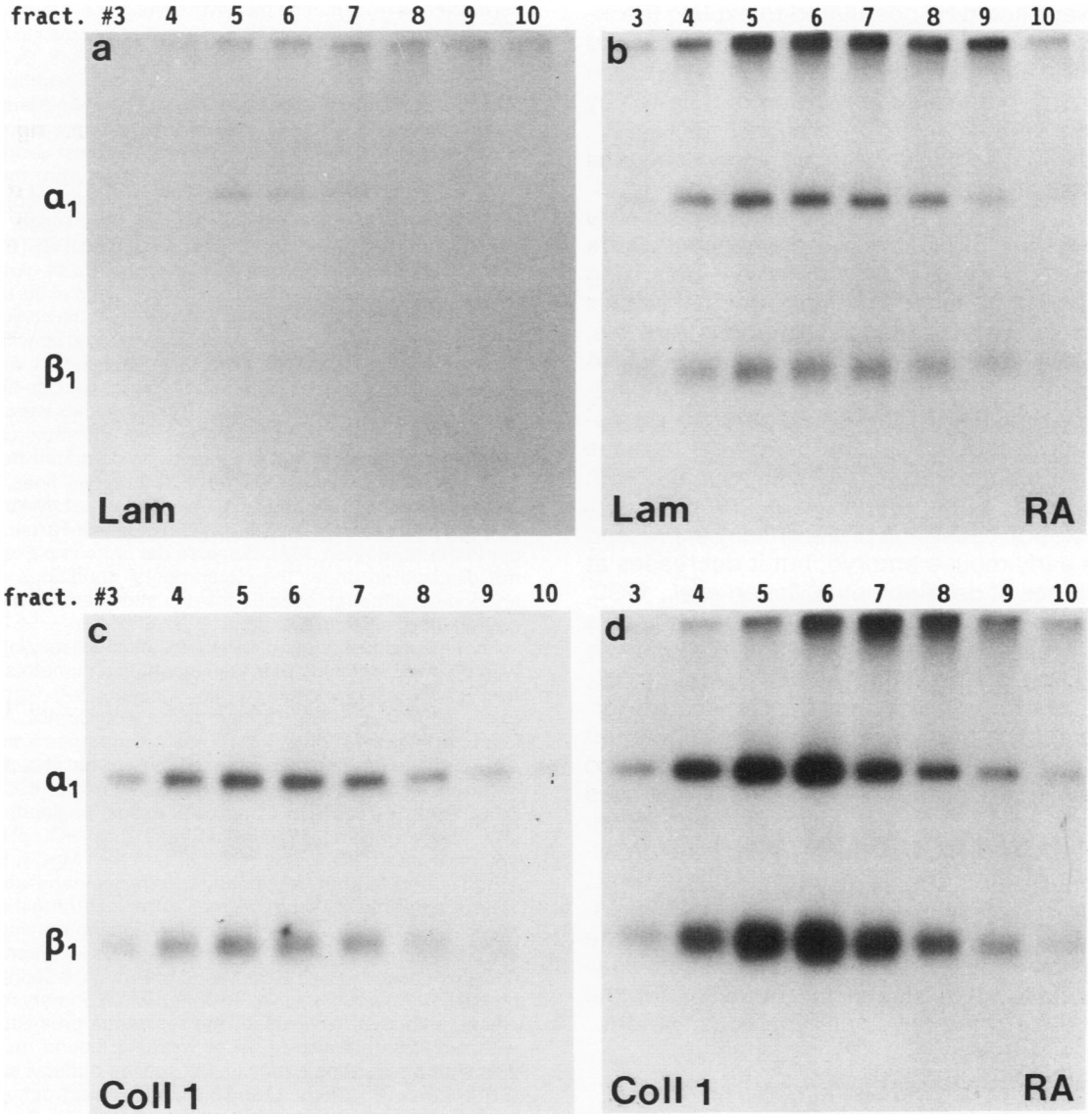

Figure 7. **Isolation of $\alpha 1/\beta 1$ integrin of SY5Y cells from laminin and collagen type I affinity columns.** SY5Y cells untreated (left) or treated with 1×10^{-5} M RA for 7 d (right) were surface-labeled with ^{125}I and lactoperoxidase. Detergent extracts were divided in two identical aliquots and applied on a laminin-Sepharose (Lam) or on a collagen type I-Sepharose (Coll 1) column. Bound proteins were eluted with 10 mM EDTA and the relevant fractions were analyzed by SDS-PAGE under nonreducing conditions. The fluorography of the gels is shown. Figures on top of each lane represent the fraction number. $\alpha 1$ and $\beta 1$ integrin bands are identified on the left side.

tiation, did not increase the neurite response to laminin (Figure 4) or to fibronectin and type I collagen (Rossino and Tarone, unpublished results). On the other hand, RA-treated cells showed an increased capacity to elongate neurites on all three matrix proteins (Figure 4), suggesting that RA activates specific mechanisms of response to these molecules. RA was the only agent among those tested to cause a specific up-regulation of $\alpha 1/\beta 1$ integrin, and the increased expression of this receptor can explain, at least in part, the increased response to laminin substrata. However, the stimulation of neurite extension on fibronectin indicates that RA also activates other mechanisms. In fact, RA did not increase the expression of $\alpha 3/\beta 1$, the presumed fibronectin receptor in these cells (Elices *et al.,* 1991), and activation of another

pathway should be postulated to explain the increased response to this substrata. This second pathway and the up-regulation of $\alpha 1/\beta 1$ can both contribute to the extension of neurites by RA-treated SY5Y cells on laminin.

Retinoids are naturally occurring morphogens that play an important role in embryonic development. The role of RA has been extensively investigated in the limb morphogenesis where this molecule was shown to have a polarizing activity and to specify the anteroposterior axis of mesenchymal tissues development (Brockes, 1989). Several studies also indicate a role of RA in the nervous system. In fact, cellular RA binding protein (CRABP) and RA receptors are present in both the central and peripheral nervous system (Benbrook *et al.*, 1988; Rees *et al.*, 1989; Zelent *et al.*, 1989; Maden *et al.*, 1990; Mamoi *et al.*, 1990). CRABP is preferentially expressed in the early mouse embryo, but it decreases at late stages of development (Maden *et al.*, 1990; Momoi *et al.*, 1990). In addition, the Hensen's node (Hornbruch and Wolpert, 1986) and the floor plate of the neural tube (Wagner *et al.*, 1990), structures that have a role in the formation of the nervous system, were shown to synthesize retinoids and to possess polarizing activity when grafted in the chick limb bud (Wagner *et al.*, 1990). Finally, exogenous administration of RA can deeply affect the development of the nervous system (Durston *et al.*, 1989). The ability of RA to modulate the expression of a laminin receptor involved in neurite extension in cultured neuroblastoma cells may thus represent a mechanism relevant for the RA activity in the developing nervous system.

Materials and methods

Cells, culture and treatments

The human neuroblastoma cell lines SK-N-SH clone SY5Y (Biedler *et al.*, 1973) and IMR32 (Tumilowicz *et al.*, 1970) were obtained, respectively, from the stock of Dr. J. Biedler (Sloan Kettering, NY) and from Dr. E. Sher (Department of Pharmacology, University of Milano, Italy). Cells were cultured in RPMI medium supplemented with 10% fetal calf serum and antibiotics. NGF 2.5S purified from mouse salivary glands was kindly provided by Dr. L. Callegaro (Fidia Pharmaceutical, Padova, Italy). Cells were treated with 100 ng/ml NGF in RPMI medium with 10% fetal calf serum. Bovine insulin (Sigma, St. Louis, MO) was used at 1 μg/ml. PMA was from Sigma and was used at 1.6×10^{-8}M. RA (all-trans retinoic acid, Sigma) was used at 1×10^{-5}M. In standard conditions, cells were treated for 7 d and the differentiating factors were renewed every 3 d by changing the medium.

Antibodies and immunoprecipitation

The polyclonal antibody to the human fibronectin receptor (FN-R), was prepared by immunizing a goat with affinity-purified FN-R from human placenta (Pytela *et al.*, 1987). The antiserum was previously characterized and shown to react with both $\beta 1$ and $\alpha 5$ subunits (Conforti *et al.*, 1989; Defilippi *et al.*, 1991). Its reactivity with $\beta 1$ allows the identification of all receptors shearing this subunit. The polyclonal antisera specific for the $\beta 1$ or the $\alpha 3$ integrin subunits were prepared in our laboratory by immunizing rabbits against synthetic peptides reproducing amino acid sequences from the cytoplasmic domains of each subunit. The following peptides, obtained from Multiple Peptide System (San Diego, CA), were used: $\beta 1$, CTTVVNPKYEGK and $\alpha 3$, CRIQPSETERLTDDY. Peptides were coupled to hemocyanin with glutaraldehyde (approximate peptide/carrier molar ratio of 50:1) and rabbits were repeatedly injected with 500 μg of the conjugate in complete Freund adjuvant. Antibodies reacted specifically with the peptide sequence used for immunization, as determined by enzyme-linked immunoassay on peptide-bovine serum albumin (BSA) conjugates. The antibodies were specific to the appropriate subunit and did not show cross-reaction with other integrin subunits as demonstrated by immunoprecipitation assays on different cell lines. The mouse monoclonal antibody to human integrin $\alpha 1$ (MAb TS2/7) was a kind gift from Dr. Martin Hemler (Dana-Farber Cancer Institute, Boston, MA). Because the α/β complexes are not dissociated under the experimental conditions used, antibodies, although specific for one subunit, immunoprecipitate both of them.

For immunoprecipitation, cells were metabolically labeled with ^{35}S-methionine by 6 or 15 h incubation in methionine-free medium (Flow Laboratories, McLean, VA) with 5% serum and 40 μC/ml of ^{35}S-methionine (800 Ci/mM, Amersham, Arlington Heights, IL). To analyze integrins from cell extracts, labeled cells were washed with ice cold phosphate-buffered saline (PBS) and extracted for 20 min at 4°C with 0.5% Triton X-100 (BDH Chemicals, Essex, England) in 20 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.4, 150 mM NaCl (TBS) with 1 mM $CaCl_2$, 1 mM $MgCl_2$, (TBS-Triton buffer) 10 μg/ml leupeptin, 4 μg/ml pepstatin, and 0,1 TIU/ml aprotinin (all from Sigma). After centrifugation at 10 000 $\times$ *g* for 10 min, extracts were incubated with the specific antibodies for 1 h at 4°C with gentle agitation. Soluble immunocomplexes were bound to Protein A-Sepharose beads (Pharmacia, Uppsala, Sweden); when primary monoclonal antibodies were used, the resin was preincubated with rabbit anti-mouse Ig. After washing, bound material was eluted by boiling beads in 1% sodium dodecyl sulfate (SDS) (Pierce, Rockford, IL) and analyzed by electrophoresis.

Pulse-chase experiment

For pulse-chase experiments, monolayers of untreated and RA-treated SY5Y cells were washed once with PBS and starved for 2 h in methionine-free medium in 5% fetal calf serum. The cells were pulse-labeled for 15 min with 200 μCi/ml of ^{35}S-methionine in methionine-free medium, washed once to remove the excess of free radioactivity, and chased for different times in unlabeled normal medium in the presence of 150 mg/l of cold L-methionine. At the indicated times, the cells were washed with cold PBS and detergent extracted. Integrins were immunoprecipitated as described above. Before immunoprecipitation the incorporated counts were determined by trichloroacetic acid precipitation of the detergent extract. Samples containing equal amounts of counts were then immunoprecipitated to allow direct comparison among different samples.

Electrophoretic analysis of proteins

SDS-polyacrylamide gel electrophoresis was performed under nonreducing conditions. Gels were made of 6% acryl-

amide using the protocol described by Laemmli (1970). Gels were processed for fluorography (Chamberlain, 1979), dried, and placed in contact with an Amersham MP Hyperfilm. ^{14}C-labeled molecular mass markers (Amersham, UK) were: myosin (200 kDa), phosphorylase B (93 kDa), BSA (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), and lysozyme (14 kDa).

Cell adhesion assays

Laminin purified from Engelbreth-Holm-Swarm (EHS) tumor was kindly provided by Dr. R. Timpl (Max Planck Institute, Martinsread, Germany). The laminin preparation did not contain detectable amounts of collagen IV as judged by amino acid analysis. Fibronectin was purified from human plasma by gelatin affinity chromatography as described (Engvall and Ruoslahti, 1977). Collagen type I was purchased from Serva (Heidelberg, Germany).

Neurite extension assays were performed as described previously (Rossino *et al.*, 1990). Multiwells plates (24 wells, Costar, Cambridge, MA) were coated with 10 μg/ml of the adhesive proteins and postcoated with BSA. Cells were detached from the culture dishes by incubation in PBS with 1 mM EGTA and washed twice in serum-free RPMI by centrifugation. Cells (1×10^4/well) were plated on coated dishes in serum-free RPMI medium for 2 h at 37°C. Adherent cells were fixed with 3% paraformaldehyde, stained with crystal violet, and photographed under phase contrast. Neurites from 150 cells were measured for each sample. Only processes longer than 15 μm (~1 cell diameter) were considered. Because cell aggregation obscured neurites, aggregates were not scored.

To test inhibition by anti FN-R serum, adherent cells were incubated with antibodies according to the following protocol. Cells treated for 7 d with RA or untreated control cells were harvested with EGTA and allowed to adhere for 15 h on microtiter wells coated with laminin (10 μg/ml), fibronectin (10 μg/ml), collagen type I (10 μg/ml), or polylysine (100 μg/ml) in medium without serum. Cells were then incubated for 2 h at 37°C with serial dilutions of the goat antiserum to human FN-R or of the preimmune serum. After washing, adherent cells were fixed, stained, and counted.

Isolation of the laminin and collagen receptors

Purified laminin was coupled to CNBr-activated Sepharose (Timpl *et al.*, 1987) yielding ~1.7–2.3 mg protein bound to 1 g dry adsorbent. The collagen type I-Sepharose matrix was prepared by coupling 2 mg of protein per gram of dry Sepharose after the procedure indicated by the manufacturer (Pharmacia-LKB). Columns were prepared from 5 ml of swollen protein adsorbent.

SY5Y cells cultured in five 80-cm^2 plates were either untreated or treated with RA as described above. Cells detached with EGTA treatment were collected in PBS and radiolabeled with 1 mC of ^{125}I as described (Tarone *et al.*, 1982). Labeled cells were extracted with 4 ml of 20 mM Tris-HCl, pH 7.4, 150 mM NaCl (TBS) with 200 mM β-octylglucoside, 1 mM $MnCl_2$, 10 μg/ml leupeptin, 4 μg/ml pepstatin, and 0.1 TIU/ml aprotinin. After centrifugation at $10\,000 \times g$, the soluble cell extract was divided in two identical aliquots and applied on the laminin and collagen affinity columns. After incubation for 1 h at room temperature, the columns were washed with 5 volumes of 50 mM β-octylglucoside in TBS plus 1 mM $MnCl_2$ and eluted sequentially with 2 volumes of 10 mM EDTA in TBS, 50 mM β-octylglucoside, and 2 volumes of 0.1 M sodium carbonate, pH 11, 50 mM β-octylglucoside. The column flow was kept at 40 ml/h through all steps of the chromatography. The peak fractions were identified by counting the radioactivity and aliquots of each fraction were concentrated by lyophilization and analyzed by electrophoresis.

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