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Modulation of $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_3\beta_1$ integrin heterodimers during human neuroblastoma cell differentiation

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Two human neuroblastoma cell lines, LAN-5 and GI-CA-N, have been analyzed for their capability to adhere to different extracellular matrix (ECM) components. The GI-CA-N cells adhered to all the tested substrates: laminin (LN), type I and type IV collagen (Coll I, Coll IV), vitronectin (VN), and fibronectin (FN). Conversely LAN-5 cells weakly attached to FN and VN, whilst adhesion on LN and Coll I and IV was strong and induced a rapid elongation of cell processes. By means of RT-PCR and immunoprecipitation we showed that the integrin pattern of these two lines was different and could explain their diversity in adhesion capability. Both cell lines express a large amount of the β_1 integrin subunit, associated with different α chains, probably responsible for their adhesion to some ECM proteins. After treatment of LAN-5 cells with biological differentiating agents, such as γ-interferon, alone or in combination with tumour necrosis factor-α (TNF-α), or retinoic acid, the levels of $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_3\beta_1$ integrin expression were enhanced, while the amount of α_v remained constant. In contrast, treatment of LAN-5 cells with TNF-α, that did not induce any maturation, or starvation in 2% foetal calf serum, that inhibited cell proliferation without affecting neural differentiation, did not induce any change in the integrin assessment. Messenger-RNAs for the two α_6 isoforms, A and B, were present in both cell lines. However, in LAN-5 cells, the protein product was neither detectable nor inducible by differentiation. Our results confirm the specific modulation of the $\alpha_1\beta_1$ integrin expression in human neuronal development, and show, for the first time, the involvement of $\alpha_2\beta_1$ and $\alpha_3\beta_1$ heterodimers in this maturational process.

Integrin; Neuroblastoma; Cell differentiation; γ-Interferon; Tumour necrosis factor-α; Retinoic acid

1. INTRODUCTION

Integrins are a complex family of heterodimeric membrane glycoproteins that mediate cell-to-cell and cell-to-extracellular matrix (ECM) interactions [1–3]. They play critical roles during development and cell differentiation [4] and are involved in tumorigenic processes such as invasion and metastasis [4,5]. In the nervous system integrins are the major class of adhesion receptors and mediate the differentiating effects of ECM components on neural cells during development [6]. Among the ECM proteins, laminin (LN) has an important role in promoting neurite outgrowth and extension in several nerve cell types [6,7]. Neural cells recognize LN through integrin receptors [8]. The $\alpha_1\beta_1$ and $\alpha_3\beta_1$ laminin receptors have been identified in cultures of neuronal cells showing support neurite extension [9,10]. Moreover, $\alpha_1\beta_1$ expression is up-regulated in neuroblastoma cells (NB) induced to differentiate by retinoic acid (RA) [11]. The $\alpha_6\beta_1$ integrin has been demonstrated to bind LN in the retina and to be involved in the early development of the optic fiber [12]. The differential expression of the A and B isoforms of the α_6 subunit was shown to be related to various stages of maturation in mouse ES cells [13] suggesting a relationship with cell differentiation processes. Among the integrins, α_v is another subunit whose expression can be modulated by cytokines and differentiating factors in various cell types [14,15], being probably involved in cell differentiation.

NB cells are a useful model system to study neuronal differentiation [16]. These cells can be induced to differentiate to a neuronal morphology upon treatments with γ-interferon (γ-IFN) [17], RA [18], and combination of γ-IFN and tumour necrosis factor-α (TNF-α) [19]. In this paper we analyzed the adhesion properties, integrin expression, and morphological changes due to ECM-integrin interactions in the neuronal model. Moreover, the involvement of specific integrins in neuroblast differentiation has been investigated.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Human recombinant γ-IFN and human recombinant TNF-α were generous gifts of Genentech Inc. (South San Francisco, CA, USA); they were stored in aliquots (30,000 IU/μl) at −80°C. RA (Sigma, St. Louis, MO, USA) was prepared at 10^{-3} M in DMSO every week and kept at −20°C. All the other reagents of biochemistry and molecular biology grade were obtained from Sigma.

2.2. *Cell cultures and treatments*

LAN-5 NB cell line was a gift of R. Seeger [20]. GI-CA-N cell line was established and characterized in our laboratory [21]. Cells were

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maintained in the logarithmic phase of growth in 75 cm^2 plastic culture flasks (Costar, Cambridge, MA, USA) in RPMI 1640 medium (Seromed, Biochrom K.G., Berlin, Germany), supplemented with sodium penicillin G (50 IU/ml), and streptomycin sulfate (50 μg/ml) (complete medium) containing 15% heat-inactivated foetal calf serum (FCS) (Seromed) at 37°C in a 5% CO_2/95% air-humidified incubator. Cells were split following treatment with 1 mM EDTA in Hank's salt solution (Seromed), washed, counted, and re-plated in fresh complete medium. Cells were treated with 1,000 IU/ml γ-IFN, or 200 IU/ml TNF, or 10^{-6} M RA, or starved in 2% FCS-complete medium for different times, as previously reported [22,23].

2.3. *Cell adhesion assay*

GI-CA-N and LAN-5 NB cells were harvested with ice-cold phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} (Seromed), resuspended in RPMI 1460 medium without FCS and seeded on 96-well plates (Costar), previously coated with different ECM substrates, respectively at a density of 1×10^5 or 2.5×10^5 cells/well. Adhesion assays were performed as previously described [13]. Briefly, wells were coated with LN, vitronectin (VN), fibronectin (FN), collagen type I (Coll I) and type IV (Coll IV) (10 μg/ml each, Sigma) or with 1% poly-D-lysine (Polyscience Inc., Warrington, PA, USA) in PBS. Coating was performed overnight at 4°C, then the plates were washed twice with PBS and incubated for 1 h at room temperature with 0.1% BSA in PBS. After two additional washes of the wells with PBS, cells were plated and incubated for 1 h at 37°C. Unattached cells were washed out with PBS and adherent cells were fixed for 5 min with 3% formaldehyde, 2% sucrose in PBS, stained with 0.5% Cresyl violet in 20% methanol for 15 min, and washed with water. After solubilization with 0.1 M citric acid in 50% ethanol (pH 4.2), A_{600} was measured by a microplate reader (Metertech, Model Σ960). The percentage of cells attached to ECM-coated cells was calculated as described by Hall et al. [24].

2.4. *RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)*

Total RNA was extracted from NB cells using the guanidine-isothiocanate and phenol/chloroform method [25]. Briefly, the guanidine suspension containing the cell lysate was phenol/chloroform extracted and the aqueous phase precipitated with absolute ethanol at −20°C. The pellet was then washed twice with 70% ethanol, dried and resuspended in sterile water. One μg of RNA was utilized for each RT-PCR amplification using a thermal cycler (Perkin Elmer Corporation, Norwalk, CT, USA) as previously described [26]. The specific primers for α_1, α_2, α_3, α_4, α_5, α_6, α_v, β_1, β_3, β_4, β_5,and β_6 were synthesized with an Applied BioSystem Oligonucleotide Syntesizer 391 following the standard procedures, on the base of the integrins cDNA published sequences. The samples were analyzed on 2% agarose gel electrophoresis containing 0.1 μg/ml ethidium bromide.

2.5. *Cell surface iodination and immunoprecipitation*

Cells were grown up to five days in the presence or absence of the various differentiation-inducing agents, and then harvested with 0.04% EDTA. After 3 washes with PBS, cells were resuspended in PBS at a density of about 5×10^7/ml and radioiodinated with 2 mCi/ml of $Na^{125}I$ (sp. act. 100 mCi/ml; ICN, Costamesa, CA, USA) for 10 min at room temperature using 300 μg/ml of lactoperoxidase (Sigma) and 0.003% H_2O_2. After labeling, the cells were lysed with 0.5% Triton X-100, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 4 mM PMSF, 2 μg/ml Pepstatin, 2 μg/ml Leupeptin (Boehringer Mannheim Corporation, Indianapolis, IN, USA), 2 μg/ml Aprotinin (Sigma) in Tris-buffered saline for 60 min on ice. The lysates were clarified adding 1/10 of volume of Pansorbin (Calbiochem, San Diego, CA, USA) and centrifuged at 40,000 rpm for 60 min at 4°C. The supernatants were then utilized for the immunoprecipitation experiments.

The following antibodies were kindly provided by the listed investigators: rabbit polyclonal antisera β_1 cyt [11], α_2 cyt, α_3 cyt, α_5 cyt [27], G. Tarone, University of Turin, Italy; rabbit polyclonal antisera anti-α_6-A, anti-α_6-B [28], anti-β_4 6944, anti-β_6, V. Quaranta, Scripps Clinic, La Jolla, CA, USA; mouse monoclonal antibody anti-α_v LM142 [29], D. Cheresh, Scripps Clinic. The other antibodies were purchased as listed: rabbit polyclonal antibody anti-α_1 (Chemicon Temecula, CA, USA), mouse monoclonal antibody anti-α_4 (Calbiochem), mouse monoclonal antibody anti-β_3 Y2/51 (Dakopatts A/S, Glostrup, Denmark).

Antibodies were adsorbed onto protein G-agarose (Pierce, Rockford, IL, USA) by incubation for 1 h at 4°C with rotation in 10 mM Tris-HCl, pH 8.5, 150 mM NaCl, 0.5% Tween 20, 0.1% Renex, 0.01% ovalbumin, 2.5 mM NaN_3 (PORT). These immunoabsorbents were incubated overnight at 4°C with aliquots of radioiodinated cell lysates diluted in PORT, washed several times and eluted by boiling in Laemmli sample buffer [30]. Samples were analyzed by SDS-PAGE on 7.5% polyacrylamide gels, followed by autoradiography on Hyperfilm MP (Amersham Buckinghamshire, UK).

3. RESULTS AND DISCUSSION

3.1. *Adhesion properties of neuroblastoma cells*

Cell adhesion assays using GI-CA-N and LAN-5 NB cell lines showed different capabilities of these cells to attach to ECM components. GI-CA-N cells were capable of attaching in the absence of FCS to all the tested substrates (LN, Coll I, Coll IV, VN, and FN) (Fig. 1). LAN-5 cells attached well to Coll I, Coll IV, and LN, whilst they weakly adhere to VN and FN (Fig. 1). These differences in adhesion capabilities could be related to different adhesion receptor patterns in the two NB cell lines which may correspond to different stages of neural crest-derived cell maturation [20,21]. When plated on purified ECM components these NB cells assumed different morphologies, spreading on the coated plastic surface and elongating neurites (Fig. 2). In both cell lines the effects of LN, Coll I and Coll IV on neurite outgrowth was evident, confirming previous results in pheocromocytoma [31,32] and after NB models [11].

3.2. *Integrin patterns of LAN-5 and GI-CA-N cells*

Integrin expression of both NB cell lines was analyzed at the mRNA level by means of RT-PCR amplifi-

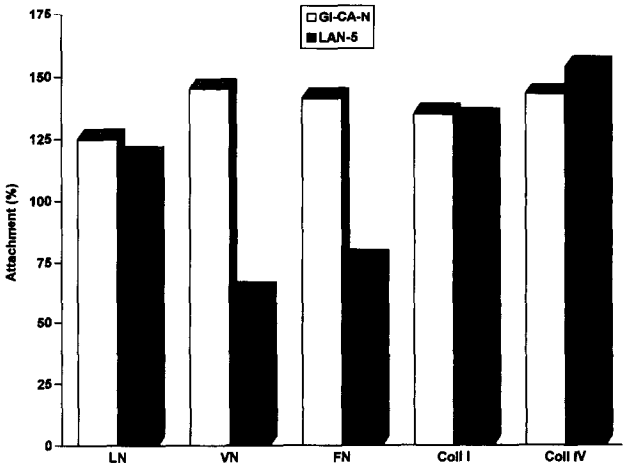

Fig. 1. Adhesion properties of GI-CA-N and LAN-5 NB cells on different ECM proteins. The data are expressed as percentage of the O.D. obtained by cells attached to poly-D-lysine and are representative of a typical experiment of three, each done in duplicate.

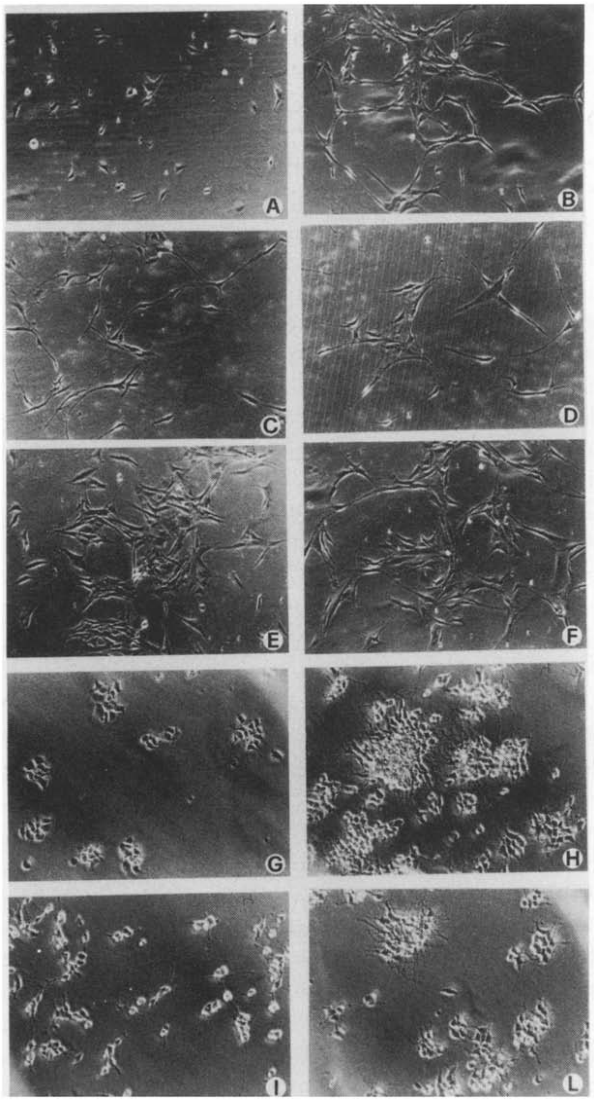

Fig. 2. Morphologies of GI-CA-N (A–F) and LAN-5 (G–L) NB cells on different ECM proteins after 3 h of adhesion in the presence of RPMI 1640 without FCS. A,G = poly-D-lysine; B,H = Coll IV; C,I = Coll I; D,L = LN; E = VN; F = FN.

cation of total RNA using subunit-specific synthetic oligonucleotides, and at the protein level by immunoprecipitation of surface radioiodinated cell lysates, using specific anti-integrin antibodies (Table I). As expected from the adhesion results, the integrin pattern of the two lines was different. Both expressed β_1 at high levels, but it associated with different α chains in GI-CA-N and LAN-5 cells. In particular, the absence of α_4 and α_5 subunits in LAN-5 cells could explain the weaker adhesion to FN, compared to GI-CA-N cells (Fig. 1), which express these integrin subunits (Table I). For the α_6 subunit we performed PCR amplifications using primers designed on the sequence codifying for the cytoplasmic portion of this molecule, which presents two different isoforms: α_6A and α_6B [27]. In LAN-5 cells the α_6B, described in mouse embryonic stem cells as the typical form of immature stages [13], was prevailing. This could reflect the low stage of maturation of LAN-5 cels according to the NB cell classification by Ciccarone et al. [33]. Moreover, the adhesion of GI-CA-N and LAN-5 cells to LN seems to be mediated by different receptors, since the $\alpha_6\beta_1$ LN receptor was highly expressed in GI-CA-N and not expressed in LAN-5 cells which might adhere to LN through $\alpha_1\beta_1$, $\alpha_2\beta_1$ or $\alpha_3\beta_1$ (Table I). The last three molecules could be responsible also for LAN-5 adhesion to Coll I and Coll IV. However, the presence in this cell line of mRNAs codifying for the α_4, α_6 and β_4 subunits, in the absence of the corresponding protein products (Table I), suggests the existence of post-transcriptional regulation mechanisms for the expression of such integrin subunits.

3.3. *Involvement of integrin expression in LAN-5 cells differentiation*

To elucidate the possible implication of these integrins during neuronal maturation we analyzed the changes in their expression after differentiation of LAN-5 cells. We previously showed that γ-IFN is able to inhibit proliferation and induce neural differentiation of NB cells [17] and its effect is enhanced by combination treatment with TNF-α [19]. LAN-5 cells are highly responsive to these treatments and to the treatment with RA, acquiring a differentiated phenotype with increased expression of neurofilament and microtubule associated proteins, and showing biochemical markers of neural maturation [17–19,22,23]. We supposed that also the integrin pattern of LAN-5 cells could have been modified reflecting, or perhaps contributing to the changes in their phenotype and behavior. To test this

Table I

Expression of integrin subunits by human NB cell lines LAN-5 and GI-CA-N

Integrin subunit	LAN-5		GI-CA-N	
	PCR[a]	IP[b]	PCR	IP
β_1	+++[c]	+++	+++	+++
α_1	+	+	−	−
α_2	+	+	+	+
α_3	+	+	+	+
α_4	+	−	+	+
α_5	−	−	++	+
α_{6A}	+	−	+++	+++
α_{6B}	+++	−	+	+
α_v	++	+	++	++
β_3	+	+	+	n.d.
β_4	+	−	−	−
β_5	±	n.d.	+	n.d.
β_6	−	−	−	−

[a] data obtained by RT-PCR (n = 3).
[b] data obtained by immunoprecipitation (n = 3).
[c] − negative; ± equivocally positive; + moderately positive; ++ positive; +++ strongly positive; n.d. not done.

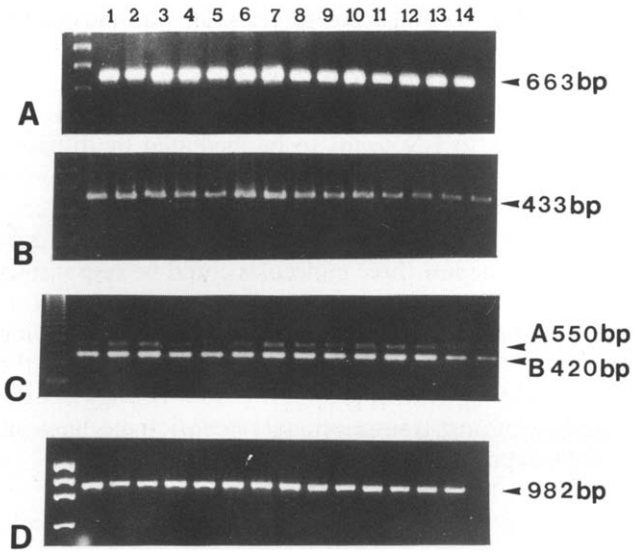

Fig. 3. Expression of β_1, α_v, α_6A and B mRNAs during NB cell differentiation. The figure shows RT-PCR amplifications of mRNAs for the integrin subunits: (A) β_1; (B) α_v; (C) α_6 A and B isoforms; (D) for the G3PDH 'housekeeping' gene. Total RNAs from untreated LAN-5 cells (lanes 1, 14), or treated with γ-IFN (1,000 IU/ml) (lanes 2–5), with RA (10^{-6} M) (lanes 6–9), and with γ-IFN + TNF-α (1,000 IU/ml, 200 IU/ml, respectively) (lanes 10–13), were extracted after 1 (lanes 2, 6, 10), 3 (lanes 3, 7, 11), 5 (lanes 4, 8, 12) and 7 days (lanes 5, 9, 13) of treatments and utilized for the RT-PCR experiments. The molecular size standards on the left are the 123 kb DNA ladder (panel A, B, and C) and the $\phi\chi$174 RF DNA–*Hae*III digest (panel D).

hypothesis, LAN-5 cells were treated with γ-IFN, γ-IFN + TNF-α, or RA, and total RNA was extracted at different time intervals and utilized for RT-PCR experiments. Oligonucleotide primers specific for β_1, α_v, and α_6 chains have been used and the results are shown in Fig. 3. Although the alternative expression of the two α_6 isoforms seems to be related to the differentiation stage of the cell, the α_6B being the typical form of immature stages in the mouse embryonic stem cells [13], we did not observe any shift between the two isoforms in all treated samples (Fig. 3C). In some experiments, a non-consistent enhancement of both isoforms was noted. No significant changes in mRNA expression were seen for β_1 and α_v subunits (Fig. 3A and B, respectively), although their expression has been shown to be regulated by various cytokines in different cell models [11,13–15]. The pattern of expression of the corresponding protein products after 5 days of treatment was then analyzed by immunoprecipitation of lysates from treated or untreated, surface radioiodinated, LAN-5 cells (Fig. 4). A significant enhancement of the $\alpha_1\beta_1$ heterodimer was seen in LAN-5 cells treated with RA, γ-IFN and, even stronger, with the combination of γ-IFN and TNF-α (Fig. 4A,B, lanes 5, 2, and 3, respectively). γ-IFN itself, or together with TNF-α enhanced the expression also of the integrins $\alpha_2\beta_1$ and $\alpha_3\beta_1$, which were not induced by RA treatment (Fig. 4C,D, lanes 2, 3, and 5). No integrin enhancement was visible when LAN-5 were treated with TNF-α alone (Fig. 4, lanes 5), which has no differentiating effect by itself on these cells [19,23]. Moreover, in cells in which the proliferation rate was arrested by starvation with 2% FCS-containing medium without affecting cell maturation [23], the amounts of such integrins (Fig. 4, lanes 6) were comparable to those seen in the untreated cells (lanes 1). These data support the relationship of the modulation of these integrins with LAN-5 neural differentiation independently from the proliferative stop itself. On the other hand, the levels of α_v and β_3 were the same in treated or untreated cells (Fig. 4E,F), suggesting that these subunits are probably not involved in neural differentiation. The anti-β_4 6944 antibody did not precipitate any protein in untreated or treated LAN-5 cells (Fig. 4H),

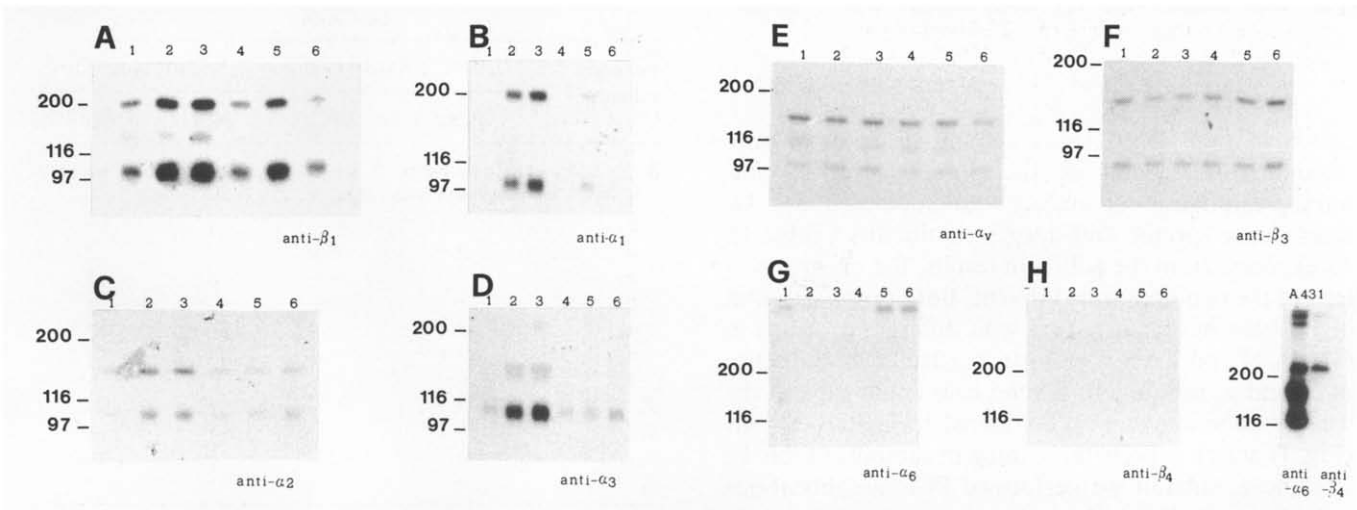

Fig. 4. Integrins expression in neuroblast differentiation induced by γ-IFN, γ-IFN + TNF-α, and RA. The figure shows immunoprecipitations of surface radioiodinated cell lysates of LAN-5 cells: untreated (lanes 1) or treated for 5 days with γ-IFN (lanes 2), γ-IFN + TNF-α (lanes 3), TNF-α (lanes 4), RA (lanes 5), at the concentration listed in the legend of Fig. 3, or starved with 2% FCS (lanes 6). In lower right panel a positive control for anti-α_6 and anti-β_4 antibodies is shown, obtained by immunoprecipitation of an A431 cell lysate. Molecular mass markers are in kilodaltons.

showing that the γ-IFN or RA-induced differentiation did not activate the transduction of the β_4 mRNA present in these cells. Also the anti-α_6A antibody (Fig. 4G) and the anti-α_6B antibody (data not shown) did not react with any protein in the treated or untreated LAN-5 cells in spite of the presence of α_6 encoding mRNA. The last finding is supported by the immunoprecipitation of β_4 and α_6 subunits in A431 cells used as a positive control (Fig. 4). In conclusion, the up-modulation of the $\alpha_1\beta_1$ integrin observed during the course of neuronal differentiation of LAN-5 cells induced by all the promoting agents, confirms previously reported data using RA on NB cells [11] and NGF on PC-12 cells [30] and points to a possible generic role for this heterodimer in neurite outgrowth [30]. In contrast, the dramatic up-regulation of the $\alpha_2\beta_1$ and $\alpha_3\beta_1$ heterodimers by γ-IFN and γ-IFN + TNF treatments, more potent differentiation-inducing protocols [19,22,23], suggests different pathways of activation for the three integrins and different functional roles in neuronal cell maturation. Although preliminary results seem to indicate that treatment of NB cells with these differentiating agents induces enhanced adhesion and neurite extension on LN, Coll I, and Coll IV (C. Rozzo, unpublished results), the question of whether the induced expression of these matrix receptors is an essential aspect of the regulation of neurite extension in NB cells is still under investigation.

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