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Developmental changes in integrin β-subunits in rat cerebral cortex

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Abstract

The present study was carried out to characterize the developmental expression of β1-, β5- and β6-integrin subunits in rat cerebral cortex. Using reverse transcriptase-polymerase chain reation, mRNA of the three β-subunits were shown to be present at all developmental stages. Based on immunoblots, β1-subunit expression was decreased during cortical development from embryonic stages to adults. β6-subunit expression appeared only in adult cortex. β5-subunit expression did not change during development. These β-subunits were expressed in cortical embryonic cells as revealed by immunological studies in vitro. β5 and β6 were also present in neuronal cells and in oligodendrocytes in adult cortex. Altogether, these results demonstrate that rat cerebral cortex expresses distinct integrin β-subunits with different developmental profiles. This switch of β-subunits may be an important mechanism for the regulation of cell behaviour during development. © 1997 Elsevier Science Ireland Ltd.

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The development of central nervous system (CNS) involves the interaction of neural cells with molecules of the extracellular matrix (ECM) [19] which affect neuronal attachment, migration, process outgrowth, and survival of both peripheral and central neurons [2]. In many cases, cells interact with ECM molecules via receptors belonging to the integrin family which is a large family of heterodimeric cell surface glycoproteins [10].

Several integrins are expressed in neurons and glial cells. They have been shown to mediate, mainly through their β-subunits, neuronal attachment and process outgrowth in vitro and to participate in morphogenetic processes in vivo [10,19]. Most integrins have been well characterized in adulthood, but little is known about expression of specific subunits and their potential role in rodent CNS development. This is of particular interest since ECM molecules are tightly regulated during these processes. In this regard, it seems important to analyze further integrin expression in CNS in vivo during embryogenesis and postnatal development.

One way to address this issue is to focus attention on the mammalian cortex since in this part of the brain, the main developmental processes occur sequentially, and are partially separated in different layers [14]. To understand the potential influence of ECM receptors in this context, a precise definition of the timing and location of integrin expression in vivo is pivotal. To this end, we have used reverse transcriptase polymerase chain reaction (RT-PCR), Western blot and immunological studies both in vivo and in vitro to determine the temporal distribution and cellular localization of specific integrin β-subunits during development of the cerebral cortex of the rat.

Wistar rats were kept in a 12:12 h dark-light cycle and received food and water ad libitum. Adult animals (4 weeks old) were killed by lethal dose of sodium pentobarbital (100 mg/kg). Fetuses 14 (E14), or 16 (E16) day old and pups postnatal day (P2) were obtained from timed-pregnant females by ceasarean section. The cortex was dissected out and either processed for cell culture or immunohistochemistry, or immediately frozen in liquid nitrogen for RNA extraction or membrane protein preparations. For

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immunohistochemistry on adult brains, rats were anesthetized with sodium pentobarbital (50 mg/kg) and perfused via the ascending aorta with paraformaldehyde 4% (w/v) in 0.1 M phosphate buffer (pH 7.4).

RNAs were extracted using the guanidine thiocyanate technique [6]. RT-PCR experiments were performed from 2 μg RNA, using specific primers of which the design was based on the published human sequences [1,18,20]. The sequence amplified for β1 was from nucleotide 2062 to 2277, for β5 from 1507 to 2203 and for β6 from 883 to 1620. Adult muscles were used as positive control for β6-subunits. For β1- and β5-subunit expression, plasmids containing cDNA fragments were used as positive controls [1,18]. Negative controls were performed by omitting cDNAs, no signal was obtained. After 30–45 cycles, sizes of the amplification products were 216, 697 and 736 bp for β1-, β5-, and β6-subunits, respectively. A primer pair for β-actin cDNA was used as control. β-actin transcripts were present in each cases. The particular primer set was such that it spanned the first intron of the rodent β-actin gene [16], in order to detect possible genomic DNA amplification, no signal was observed at 330 bp. The amplified products were size-fractionated on a 1% (w/v) agarose gel containing ethidium bromide.

Cerebral cortices from each different stages were homogenized by passing through a 25 gauge needle, in lysis buffer (0.15 M sucrose, 25 mM HEPES, 3 mM DTT, 0.5 μg/ml leupeptin and 190 mM PMSF). Membranes were collected by ultracentrifugation. Adult lung, liver and muscles, were used as positive controls for β1-, β5-, and β6-subunits, respectively. Protein concentrations were determined by Bradford's technique [3]. Western blot analysis were performed with 100 μg proteins. Blots were blocked overnight at 4°C in buffer containing 5% milk and 2% bovine serum albumin (BSA), and then incubated successively with a specific antibody to integrin β1- (1:1000; kindly provided by Dr. R. Hynes) [13], β5- (1:500; Chemicon) or β6-subunit (1:2000; generous gift from Dr. V. Quaranta) [20], and with an HRP-linked anti-rabbit antibody (1:2500; Amersham) for 1 h at room temperature. Detection was performed using ECL kit (Amersham).

Brains from each different stages were removed, post-fixed for 3 h, and cryoprotected in a 15% sucrose solution. Cryosections (25 μm) were thaw-mounted onto glass slides coated with Vectabond (Vector). Following a pretreatment with H_2O_2 to quench endogenous peroxidase activities, tissue sections were incubated in rabbit polyclonal antibodies to β1-, β5-, or β6- integrin subunits (1:1000, 1:500 and 1:1500, respectively) in phosphate buffer containing 3% BSA overnight at 4°C. These sections were then processed according to Davidoff and Schultze [7]. The peroxidase activity was revealed with the diaminobenzidine-hydrogen peroxide technique (Vector). Controls of the histological procedure were performed either by omitting the first specific antibody to integrin β-subunit, or by incubating tissue sections with a preimmune serum.

Cultures were prepared from embryonic E13 cortex as previously described [21]. After 3–4 days in vitro, cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.5) for 15 min, washed several times in PBS, and incubated in the first antibody solution containing 0.1% Triton X-100 and 10% normal goat serum. Immunostaining was performed on cultures using rabbit polyclonal antibodies to β1-, β5- and β6-subunits, at the dilutions of 1:750, 1:400 and 1:1500, respectively. After overnight incubation at 4°C, cultures were washed in PBS and incubated in biotinylated anti-rabbit antibody (1:250; Vectastain, Vector) for 1 h at room temperature and again washed several times in PBS. The biotinylated secondary antibodies were detected using Texas Red-coupled streptavidin (1:200; Vector). After several washes in PBS, cultures were embedded in a glycerol containing medium (Aqua-Poly/mount, Polysciences). Non-specific staining was obtained by omitting the first incubation in specific antibodies.

A single band was detected with each primer pair with the expected size using cerebral cortical tissue, plasmid DNA encoding a cDNA fragment for β1- and β5-subunits [1,18], or cDNA from muscle for β6-subunit. Each of these results have been reproduced several times. The three β-subunits were detected in mRNA from E14, E16, P2, and adult cortex (data not shown). The identity of the RT-PCR products was confirmed either by sequencing (for the β1-subunit) or restriction fragment analysis with a number of appropriate enzymes (for β5 and β6).

Each antibody recognized a single band of the appropriate size on immunoblots (Fig. 1). The anti-β1 serum gave a strong band at both embryonic ages, but no product was detectable at P2 or in the adult. β5 was detected throughout cortical development by immunoblot. β6 gave a result that was the converse of β1. It was not detected in the embryo, gave a weak band at P2, but was strongly present in the adult. These developmental profiles were confirmed by

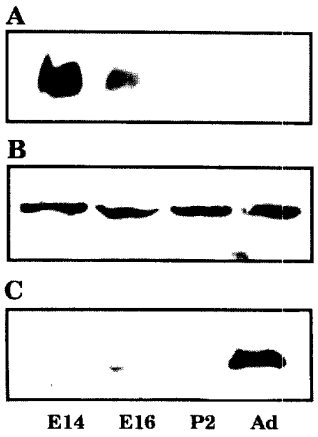

Fig. 1. Detection by Western blotting of β-integrin subunits ((A) β1; (B) β5; (C) β6) in rat cerebral cortex at different developmental stages, embryonic day 14 (E14), embryonic day 16 (E16), postnatal day 2 (P2) and adult (Ad) cortices.

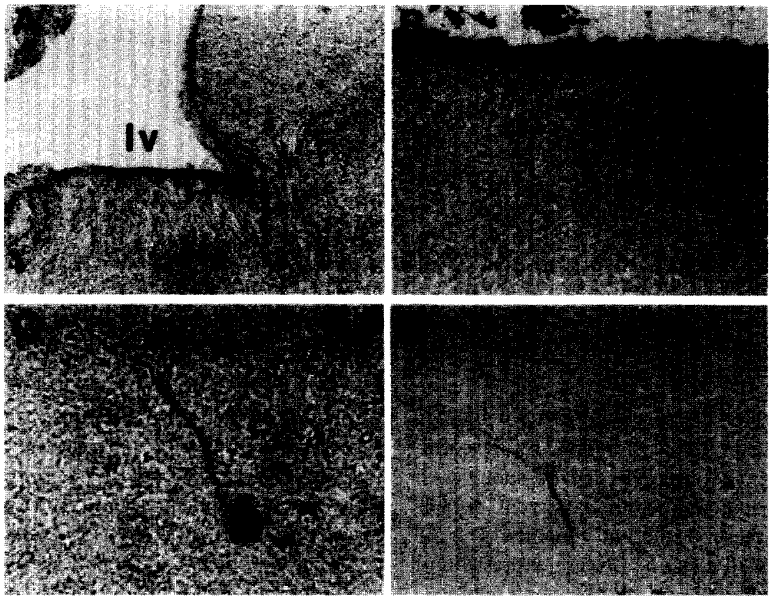

Fig. 2. Light microscopic localization of β1 (A), β5 (B,D) and β6 (C) -integrin subunits in the rat brain at embryonic day 14 (A), postnatal day 2 (B) or in the adult (C,D) revealed by the immunoperoxidase technique of Davidoff and Schulze [7]. The arrows point to the stained cells which were mainly neurons. Scale bars, (A,B) 450 μm; (C) 85 μm; (D) 125 μm.

immunostaining of fetal, postnatal and adult rat brain with each of the three subunit-specific antibodies (Fig. 2). In the adult, while we could not detect a signal with the β1, we were able to detect both β5 and β6 in both neurons and oligodendrocytes of the white matter (Fig. 3). In both cases the staining appeared to be cytoplasmic or perinuclear in both cell types, and was concentrated in the perikarya.

In 3–4 day cultures from E13 cerebral cortex, the three anti-β antiserum gave largely similar results (Fig. 4). In each case virtually every process-bearing cell was stained. The small percentage of unstained cells was entirely composed of cells with a fibroblastic morphology. Previous experiments have shown that in these cultures, the process-bearing cells are mostly undifferentiated neural precursor cells plus a small number of differentiated neurons [21]. The cells with a fibroblastic morphology are predominantly fibro-

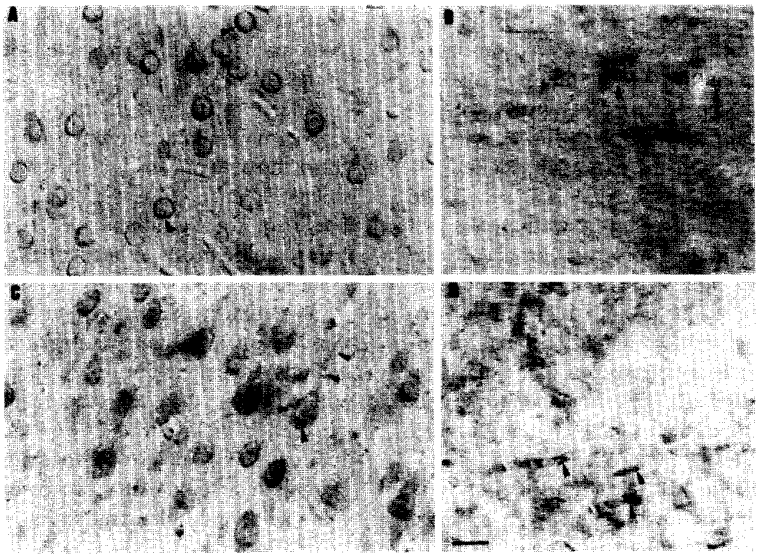

Fig. 3. Integrin β5 (A,B) and β6 (C,D) -subunit immunoreactive structures in adult rat brain, revealed by the immunoperoxidase technique of Davidoff and Schulze [7]. The staining was present in neurons of cortical layers (A,C) and in oligodendrocytes of white matter (B,D). The arrows point to the stained cells. Scale bar, 85 μm.

Fig. 4. Integrin β-subunit immunofluorescence in cell cultures prepared from E13 cortices. The staining was performed with β1 (A), β5 (B) and β6 (C) antibodies. Note that both cell bodies and processes were immunoreactive. Scale bar, 100 μm.

blasts and endothelial cells. These young cultures contain virtually no differentiated glial cells. This result corresponds well to the data regarding β1- and β5-subunits. It confirms that the embryonic neuroepithelial precursor cells are indeed expressing these β-integrin subunits. The β6 result corresponds less well since no protein expression of this subunit was detected embryonically by immunoblots.

Two major conclusions emerge from this study. First, all three β-subunits are expressed in the CNS but with different patterns of developmental regulation. This suggests that each is involved in a different set of cellular and developmental functions. Second, unlike β1-, the β5- and β6-subunits are expressed in adult neurons and oligodendrocytes, and probably have, therefore, a role in the physiology of these cells.

Our finding that β1-subunit protein content is down-regulated during cortical development is in accordance with general agreement that this subunit is expressed from the earliest stages of development [12,17]. This could be due to a developmental decrease in distal promoter activity of β1-subunit gene [9]. In chick and quail embryos, a loss of both laminin and fibronectin receptors was observed on neural tube cells during development [8]. Furthermore, fibronectin is abundant in the preplate before afferents grow in. Similarly, laminin also appears transiently in the developing brain [19]. This correlates with the expression of the β1-subunit that we have observed. Although in both studies the ECM receptor was not characterized, it is well known that among the β1-subunits are receptors for fibronectin and laminin [10]. In cortical cultures, we found β1 immunoreactivity in embryonic cells. This is consistent with in vitro studies showing a staining with β1-antibody in undifferentiated oligodendrocytes, and a decrease in this expression during the cell differentiation [15,17]. Altogether, these data are consistent with a specific role of the β1-integrin in cell maturation and motility during embryogenesis.

The β5-subunit expression has been mostly studied in vitro, and our immunological study revealed that it is indeed expressed in embryonic cells in culture. Previous data indicate that β5-subunit is expressed in oligodendroglial primary cells [15]. We failed to detect any variation in β5-subunit expression during development, although an up-regulation upon oligodendrocyte differentiation has been described in vitro [15]. These could be explained by differences between in vivo and in vitro studies. Differentiation and/or development are not only associated with changes in integrin expression, but also with regulation of receptor function or affinity, probably involving changes in receptor conformation [10]. Although we cannot exclude any additional modification of β5-integrin function, this subunit could have different function at early and late stages of development, by interacting with different ECM molecules.

A new result is the detection of β6-subunit in cortical layers of adult brain, this expression being developmentally regulated. So far, only a few studies have focused on β6-integrin expression. β6-subunit is expressed in cortical embryonic cells in culture while this subunit could not be discerned biochemically in vivo. This is not the first example of cells expressing more integrin subunits in culture than in vivo [4]. The β6-subunit has been found so far to constitute only a fibronectin receptor [5] and so it would have been expected to be down-regulated coincident with fibronectin expression. Thus, it is possible either that this subunit could bind to other ligands mainly expressed in adult cortex, or that differentiation of both neuronal and glial cells would be related to an up-regulation of this subunit.

The intracellular staining of β-subunits observed within the cytoplasm of cells is somewhat surprising for transmembrane proteins. However, the presence of an intracellular precursor for β1-subunit has been described, this protein being antigenically related to the mature form [11]. This could be also the case for β5- and β6-subunits.

In summary, this study demonstrates that rat cerebral cortex expresses distinct integrin β-subunits which are differently regulated during development. The switch of β-subunits may be an important mechanism for the regulation of cell behaviour during development. One challenge in the future will be to determine specific roles for each integrin β-subunit in developmental processes leading to generation of the mature and functional brain.

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