Expression of α1 integrin, a laminin-collagen receptor, during myogenesis and neurogenesis in the avian embryo

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Summary

In this study, we have examined the spatiotemporal distribution of the α1 integrin subunit, a putative laminin and collagen receptor, in avian embryos, using immunofluorescence microscopy and immunoblotting techniques. We used an antibody raised against a gizzard 175×10^3 M_r membrane protein which was described previously and which we found to be immunologically identical to the chicken α1 integrin subunit. In adult avian tissues, α1 integrin exhibited a very restricted pattern of expression; it was detected only in smooth muscle and in capillary endothelial cells. In the developing embryo, α1 integrin subunit expression was discovered in addition to smooth muscle and capillary endothelial cells, transiently, in both central and peripheral nervous systems and in striated muscles, in association with laminin and collagen IV. α1 integrin was practically absent from most epithelial tissues, including the liver, pancreas and kidney tubules, and was weakly expressed by tissues that were not associated with laminin and collagen IV. In the nervous system, α1 integrin subunit expression occurred predominantly at the time of early neuronal differentiation. During skeletal muscle development, α1 integrin was expressed on myogenic precursors, during myoblast migration, and in differentiating myotubes. α1 integrin disappeared from skeletal muscle cells as they became contractile. In visceral and vascular smooth muscles, α1 integrin appeared specifically during early smooth muscle cell differentiation and, later, was permanently expressed after cell maturation. These results indicate that (i) the expression pattern of α1 integrin is consistent with a function as a laminin/collagen IV receptor; (ii) during avian development, expression of the α1 integrin subunit is spatially and temporally regulated; (iii) during myogenesis and neurogenesis, expression of α1 integrin is transient and correlates with cell migration and differentiation.

Key words: avian embryo, integrin, laminin, collagen.

Introduction

Integrins are a group of non-covalently associated heterodimers of and subunits that function in cell-to-cell and cell-to-extracellular matrix adhesions (see, for reviews, Humphries, 1990; Hynes, 1992). Integrins have been classified according to their subunits, each of which may associate with different subunits. There are at least eight β1 integrins (α1β1, ..., α8β1), that are widely distributed on most cell types. β1 integrins act primarily as cell surface adhesion receptors for extracellular matrix molecules including fibronectin, laminin, collagens and vitronectin. Some β1 integrins apparently bind to a single extracellular matrix ligand, while others are more promiscuous in their ligand recognition. However, members of β1 integrins may also function as cell-cell adhesion molecules (Larjava et al., 1990; Elices et al., 1990; Marchisio et al., 1991; Taichman et al., 1991). Moreover, the ligand-binding specificity of β1 integrins may differ in various cell types at least in vitro, thus generating additional functional diversity within the family (Elices and Hemler, 1989; Languino et al., 1989).

The great diversity of integrin receptors and their versatility in ligand binding raises the problem of determining their exact function in the various adhesive processes that take place during embryogenesis and in the adult organism. Are the different integrins that bind common ligands interchangeable in every tissue or do they show tissue and functional specificities and, thus, participate in defined adhesion events? An approach to evaluate the various functions in which each integrin may play a role is to determine their expression pattern during embryogenesis and in adult tissues. β1 integrins have been found in a large variety of cell types cultured in vitro. In the adult organism, many different tissues express β1 integrins (Bozyczko et al., 1989;

Korhonen et al., 1990; Sonnenberg et al., 1990a; Virtanen et al., 1990; Terracio et al., 1991; Hertle et al., 1991) and, in the embryo, most cells express the β1 subunit of integrins (Duband et al., 1986). However, little is known about the tissue distribution and possible developmental regulation of the various α chains.

α1β1 integrin is an RGD-independent receptor for both laminin and collagens (Hemler et al., 1985; Kramer and Marks, 1989; Turner et al., 1989; Belkin et al., 1990; Ignatius et al., 1990; Rossino et al., 1990; Tawil et al., 1990; Tomaselli et al., 1990; Syfrig et al., 1991; Vandenberg et al., 1991). The α1 integrin subunit has been demonstrated on a large variety of cell types cultured in vitro, including neuronal cells (Ignatius and Reichardt, 1988; Turner et al., 1989; Rossino et al., 1990; Tawil et al., 1990; Tomaselli et al., 1990), melanoma cells (Kramer and Marks, 1989), microvascular endothelial cells (Korhonen et al., 1990; Defilippi et al., 1991), smooth muscle cells (Kelly et al., 1987; Belkin et al., 1990; Syfrig et al., 1991), cardiomyocytes (Terracio et al., 1991) and hepatocytes (Forsberg et al., 1990). In contrast, in adult human tissues, α1 integrin has been so far detected only in capillary endothelial cells and in smooth muscle cells (Belkin et al., 1990; Virtanen et al., 1990; Defilippi et al., 1991). In cultured cells, α1 integrin expression can be strongly regulated by nerve growth factor (Rossino et al., 1990), transforming growth factor-β1 (Heino et al., 1989), tumor necrosis factor, retinoic acid and phorbol esters (Defilippi et al., 1991; Rossino et al., 1990, 1991). These observations suggest that α1 integrin might be expressed transiently under the control of growth factors in a number of tissues during embryonic development and might play a critical role during cell differentiation.

In this study, we have analyzed the distribution of α1 integrin during avian development. We show that α1 integrin is expressed at high levels transiently in nervous tissues and striated muscle cells, and permanently in smooth muscle cells and capillary endothelial cells. It is practically absent from other tissues, particularly epithelia. This restricted pattern of distribution is indicative of tightly controlled developmental regulation of α1 integrin expression which correlates with defined morphogenetic events occurring during neurogenesis and myogenesis.

Materials and methods

Embryos and adult animals

Japanese quail (*Coturnix coturnix japonica*) animals were used throughout the study. Eggs were incubated at 37 ± 1°C and staged according to the number of somite pairs and the duration of incubation. Adult animals were kindly provided by Dr M.-C. Lainé (Centre de Biologie Cellulaire, Ivry-sur-Seine, France). Late embryos and adult animals were killed by decapitation.

Antibodies

Purification of a p175 membrane protein from chicken gizzard and production of rabbit antibodies directed against this molecule were described previously (Koteliansky et al., 1985). The monoclonal antibody TS2/7 against human β1 integrin was from T Cell Sciences (Cambridge, MA, USA). Polyclonal antibodies to human

β1 integrin were raised and characterized previously (Belkin et al., 1990). Purification of the α1β1 integrin complex from chicken gizzard and rabbit sera directed to the natively isolated α1 subunit were described previously (Syfrig et al., 1991). A rat monoclonal antibody, ES46, directed against the chicken β1 integrin subunit was described previously (Duband et al., 1988; Mueller et al., 1988) and kindly provided by Dr K. M. Yamada (NIH, Bethesda, MD, USA). A rabbit polyclonal antibody to mouse laminin was described previously (Duband and Thiery, 1987), and a sheep polyclonal antibody also directed against mouse laminin was a generous gift of Dr H. Kleinman (NIH, Bethesda, MD, USA). Rabbit sera to chicken collagen I and to human collagen IV were purchased from Institut Pasteur-Lyon (France). Two mouse monoclonal antibodies, NC-1, which recognizes migrating neural crest cells and a number of neural tissues, and MB-1, which recognizes quail endothelial cells, were characterized previously (Péault et al., 1983; Vincent and Thiery, 1984). Rabbit polyclonal antibodies to α-smooth muscle actin were purchased from Sigma.

Histological sections and immunofluorescent staining

Embryos were routinely fixed at room temperature in 3.7% formaldehyde in phosphate-buffered saline (PBS) for 1-8 hours, depending on the size of the embryos. After several extensive washes in PBS, embryos were embedded in a graduated series of sucrose solutions in PBS (12-18% wt/vol) and subsequently frozen in Tissue Tek (Lab-Tek Products) in liquid nitrogen. Adult tissues were directly immersed in liquid nitrogen after isolation and immediately processed for histological sectioning. Sections were cut at 7-12 μm on a cryostat (Bright Instrument Co. Ltd., Huntington, England) and mounted on slides coated with a gelatin solution. Immunofluorescent staining of sections was performed essentially as described previously (Duband and Thiery, 1987). Control stainings were performed using preimmune or non-immune immunoglobulins as primary antibodies. No significant staining was obtained with these control antibodies even in compact tissues. Sections were observed with a Leitz Orthoplan epifluorescent microscope and photographed using TMX-400 Kodak film.

Immunoprecipitation and immunoblotting analyses

Immunoprecipitation of Triton X-100-extracted proteins from human uterus smooth muscle with the monoclonal antibody TS2/7, or with a polyclonal antibody directed against both human and chicken β1 integrin was described previously (Belkin et al., 1990). For immunoblotting, samples of embryonic or adult tissues were briefly homogenized and extracted at 90°C with SDS-sample buffer under reducing conditions. The extracts were clarified by centrifugation and subjected to SDS-PAGE followed by immunoblotting analysis with antibodies to α1 or β1 integrins. PAGE was performed in Laemmli buffer system on slab 7.5% polyacrylamide minigels. The protein bands were electroblotted for 1 hour onto nitrocellulose in 50 mM Tris-glycine, 20% methanol buffer. The nitrocellulose membranes were then incubated with a 4% bovine serum albumin solution in PBS for 1 hour at 37°C, followed by incubation with antibody solution for 1 hour at room temperature. Control stainings were performed using preimmune or non-immune immunoglobulins as primary antibodies. The sheets were then rinsed in PBS supplemented with 1% Tween 20 and incubated with ^{125}I-labelled protein A (Amersham) for 1 hour at room temperature. After rinsing, the blot was subjected to autoradiography. For immunodepletion experiments, 14-day-old embryonic gizzards were extracted in 2% Triton X-100 in PBS supplemented with 1 mM Ca^{2+} and Mg^{2+} and 3 mM phenylmethylsulfonyl fluoride. Lysates were clarified by centrifugation at 11,000 **g** and mixed three times successively with rabbit

serum to the avian 1 integrin subunit or with antibodies to p175 protein at the final dilution of 1/100 followed by incubation with protein A-Sepharose (Sigma) and centrifugation. The samples were then subjected to SDS-PAGE followed by immunoblotting analysis with antibodies to p175 protein or to the avian 1 integrin subunit.

Results

Identification of the polyclonal anti-chicken p175 membrane protein antibody as an anti-chicken α1 integrin antibody

In a previous study, a p175 membrane protein was isolated from chicken gizzard smooth muscles (Koteliansky et al., 1985). The tissular origin, surface localization and biochemical properties of this protein raise the possibility that it may be the integrin 1 subunit. The specificity of rabbit antibodies against the p175 protein was analyzed using several different techniques. First, human uterus smooth muscle membrane extracts were immunoprecipitated either with a monoclonal antibody to human 1 integrin (TS2/7) or a previously-characterized polyclonal antibody against human uterus smooth muscle 1 integrin (Belkin et al., 1990), followed by immunoblotting with antibodies to the p175 protein. As shown in Fig. 1A, both antibodies precipitated a dimer of proteins of approximately 110×10^3 and 195×10^3 M_r corresponding to the 1 and 1 subunits of the

human VLA-1 integrin, respectively. On the corresponding immunoblots, the polyclonal antibody to p175 protein specifically stained the upper band of VLA-1 (Fig. 1A). Conversely, antibodies to the p175 protein immunoprecipitated a dimer of proteins of 110×10^3 and 195×10^3 M_r from human uterus smooth muscle protein extracts, and the 195×10^3 M_r band was recognized on immunoblots by the polyclonal antibody to the human 1 integrin subunit (data not shown). Second, immunoblotting of whole extracts of human uterus smooth muscles with the antibody to p175 protein revealed a single band of about 195×10^3 M_r and a protein of approximately 175×10^3 M_r on adult chicken gizzard extract (Fig. 1B). In addition, the antibody to p175 protein recognized on immunoblots the 1 subunit of purified avian 1 1 integrin complex (Fig. 1B). Finally, in order to rule out the possibility that the antibody to the p175 protein might also recognize another integrin subunit comigrating with the 1 integrin, we performed immunodepletions experiments. First, chicken gizzard extracts were immunodepleted with a recently characterized rabbit antiserum to avian 1 integrin (Syfrig et al., 1991), followed by immunoblotting with the anti-p175 antibody. As shown in Fig. 1C, the antibody to p175 did not detect any band at 175×10^3 M_r after immunodepletion with the antiserum to avian 1 integrin. Conversely, antibodies to 1 integrin subunit did not detect any band after immunodepletion with the antibody to p175 (data not shown). These results indicate that the anti-p175 protein antibody specifi-
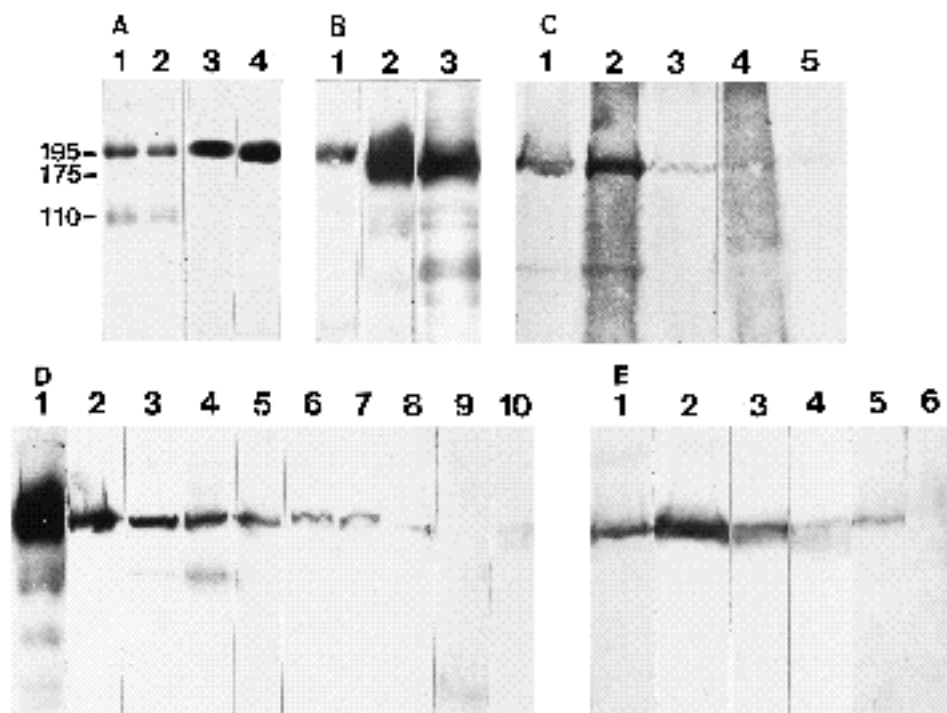
Fig. 1. Analysis of the specificity of antibodies against chicken p175 protein. (A) Crude Triton X-100 protein extracts from human uterus smooth muscle were immunoprecipitated using the monoclonal antibody TS2/7 against human 1 integrin (lane 1) or polyclonal antibodies against human 1 integrin coupled to Sepharose 4B (lane 2). Immunoprecipitates were analyzed by SDS-PAGE and protein bands were visualized by Coomassie blue staining (lanes 1, 2) or were subjected to immunoblotting with polyclonal antibodies to chicken p175 protein (lanes 3, 4). Positions of human 1 (195×10^3) and 1 integrin subunits (110×10^3) and of avian 1 (175×10^3) integrin are denoted at the left of the gel. (B) Immunoblots of extracts of human smooth muscle cells (lane 1), adult avian gizzard (lane 2) and avian 1 1 integrin complex purified from adult chicken gizzard (lane 3) with antibodies to p175 protein.

(C) Immunodepletion of avian gizzard extract with a rabbit antiserum directed against avian 1 integrin followed by immunoblotting with antibodies to p175 protein (see Materials and methods). (Lane 1) initial extract before immunoprecipitation; (lane 2) first immunoprecipitate; (lane 3) extract after the first immunoprecipitation; (lane 4) third immunoprecipitate; (lane 5) extract after the third immunoprecipitation. (D) Immunoblots of extracts of adult avian gizzard (lane 1), intestine (lane 2), aorta (lane 3), lung (lane 4), kidney (lane 5), skin (lane 6), heart (lane 7), skeletal muscle (lane 8), brain (lane 9) and liver (lane 10) with polyclonal antibodies to chicken p175 protein. (E) Immunoblots of extracts of gizzard at E7 (lane 1) and E14 (lane 2), skeletal muscles at E7 (lane 3) and E14 (lane 4) and spinal cord at E6 (lane 5) and E14 (lane 6) with polyclonal antibodies to chicken p175 protein.

Table 1. α*1 integrin expression in adult quail tissues*

Tissue type	1 integrin expression
Nervous system	
Central	
Neurons	−
Glia	−
Peripheral	
Neurons	−
Glia	−
Schwann cells	−
Muscles	
Striated skeletal muscles	−
Cardiac muscle	−
Smooth muscles	+
(visceral, vascular, pulmonary)	
Endothelium	
Arteries/veins	−
Capillaries	+
Skin	
Epidermis	−
Dermis	− (except smooth muscle)
Digestive tract	− (except smooth muscle)
Liver	−
Pancreas	−
Lung	− (except smooth muscle)
Kidney	− (except mesangial cells)

cally recognized the 1 integrin subunit in both human and avian species. This antibody was used for studying the spatiotemporal distribution of 1 integrin during avian embryogenesis and in the adult animal by immunofluorescence microscopy of frozen sections and by immunoblotting tissue extracts. The distribution pattern of 1 integrin was compared with those of its putative ligands, laminin and collagens.

α*1 integrin expression in adult avian tissues*

As shown in Fig. 1D, 1 integrin was present in large amounts in immunoblots of extracts from the gizzard, intestine, aorta and lung. In contrast, a fainter band corresponding to 1 integrin could be detected in extracts from the heart, skeletal muscles, skin and kidney, and no staining was found in extracts from the brain and liver. Using immunofluorescence labeling, it was found that, in the gizzard, lung, aorta and intestine, only smooth muscle cells were intensely stained for 1 integrin and that, in the other tissues examined, only capillary endothelial cells and smooth muscle cells of blood vessels showed significant immunoreactive material (Table 1). Thus, the 1 integrin expression found on immunoblots of extracts from the heart, skeletal muscles and skin could be attributed mostly to capillary endothelial cells and to vascular smooth muscle cells. The distribution of 1 integrin in adult avian tissues is summarized in Table 1. It shows that 1 integrin expression was restricted to smooth muscles and capillary endothelia.

α*1 integrin expression in the early embryo*

1 integrin immunoreactivity was detected early, during neurulation in the 6-somite-stage avian embryo. It was found in all tissues deriving from the three primary germ layers (Fig. 2A). However, staining was weak and uniform throughout the embryo. Only a few tissues occasionally were seen to express 1 integrin at higher levels; these were the notochord and the most caudal part of the growing Wolffian duct. The faint staining for 1 integrin persisted in tissues until day 2.5 of development (E2.5). By the 20- to 25-somite stage, 1 integrin distribution gradually changed to become selectively enhanced in the neural tube in cranial regions, in the most rostral myotomes, in the myocardium and in endothelial cells of developing capillaries (see below). By E3, the pattern of expression of 1 integrin became tissue specific, particularly in muscles and in the nervous system.

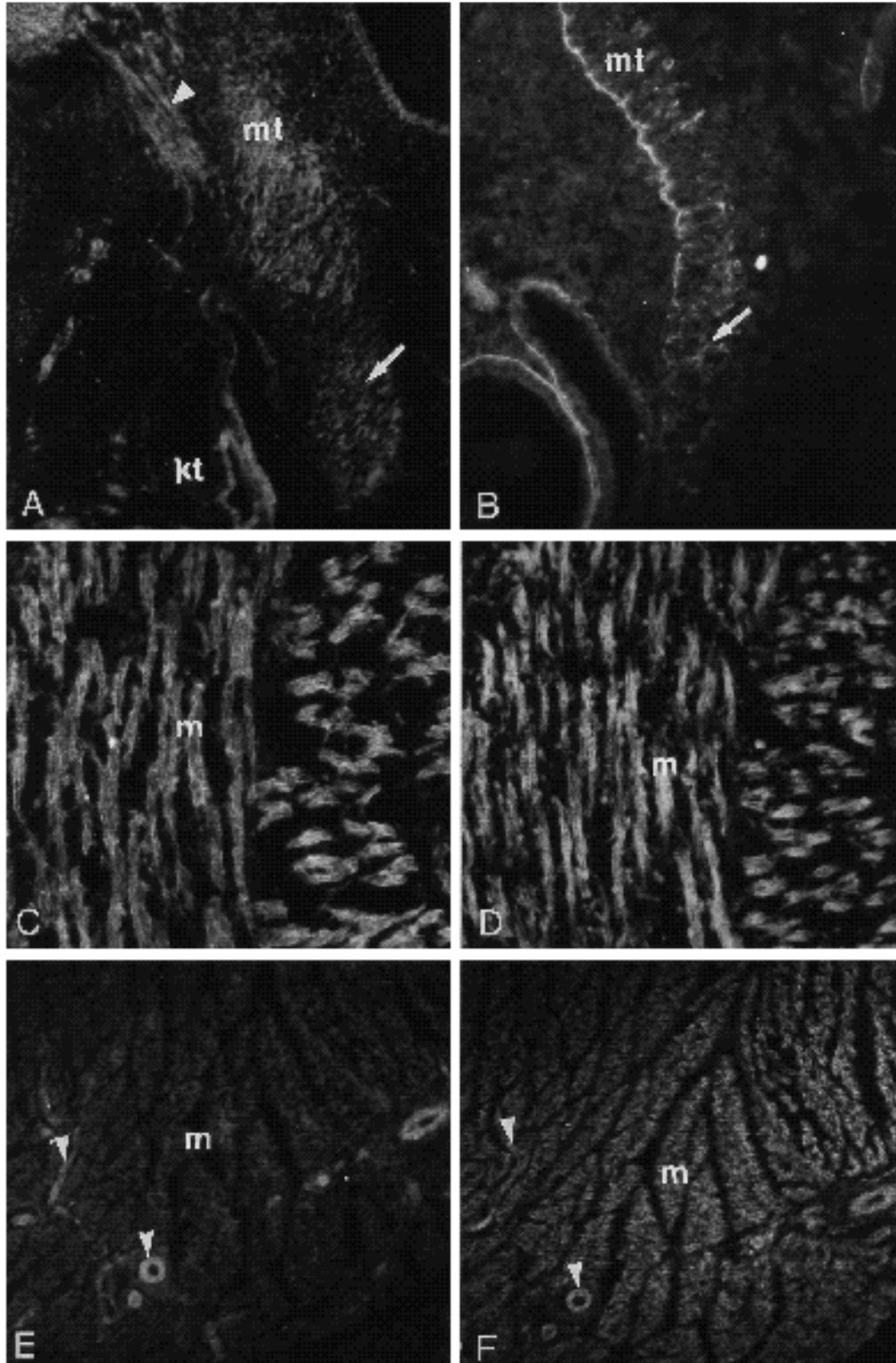### α*1 integrin expression in skeletal muscles*

The skeletal musculature in the trunk derives from the somites, an epithelial structure formed from the segmental plate located in the caudal end of the embryo. Segmental plate cells showed negligible staining for both 1 integrin (Fig. 2A), laminin and collagens I and IV (not shown, but see Duband et al., 1987; Duband and Thiery, 1987). 1 integrin staining was slightly enhanced during condensation of the segmental plate into the somitic epithelium (Fig. 2B). This increase in 1 integrin staining correlated with the appearance of laminin and collagens surrounding the somite (Duband et al., 1987; Duband and Thiery, 1987).

Shortly after their formation, somites undergo compartmentalization into the sclerotome, a mesenchyme from which the cartilage of the vertebrae develop, and the dermamyotome, an epithelial sheet from which the dermis and the musculature arise. This reorganization was accompanied by a relative decrease in 1 integrin staining among sclerotomal cells, but with no noticeable changes in the dermamyotome (not shown). Once formed, the dermamyotome is transformed into a double-layered epithelium comprising the dermatome and the myotome. Cells that constituted the myotome were strongly stained for 1 integrin as soon as they appeared (Fig. 2C). Coincidentally, a basal lamina containing both laminin and collagens I and IV was deposited along the myotome (Fig. 2D). The sequential appearance and increase in 1 integrin staining in the myotome could be detected first in the more rostral segments by approximately stage 20 somites, then appeared gradually in the most caudal segments as the development of the embryo progressed. By E4, all myotomes were formed and expressed high levels of 1 integrin. 1 integrin staining became more pronounced on the myotome as it extended towards the ventral regions of the embryo (Fig. 2E). Laminin also remained abundant along the myotome (Fig. 2F).

At E4 a number of myoblasts start to separate from the most ventral portion of the myotome and migrate within the lateral body wall to form the abdominal muscles. As shown in Fig. 3A, B, these myoblasts were in contact with a laminin matrix and expressed high amounts of 1 integrin. Myoblasts reach their target sites and form the abdominal muscle blastemas by E6. By E9-E10, myotubes have appeared and the abdominal muscles have their defined anatomy (see for details, Jaffredo et al., 1988). At this stage, 1 integrin staining was conspicuous on myotubes (Fig. 3C), and laminin immunoreactive material was also
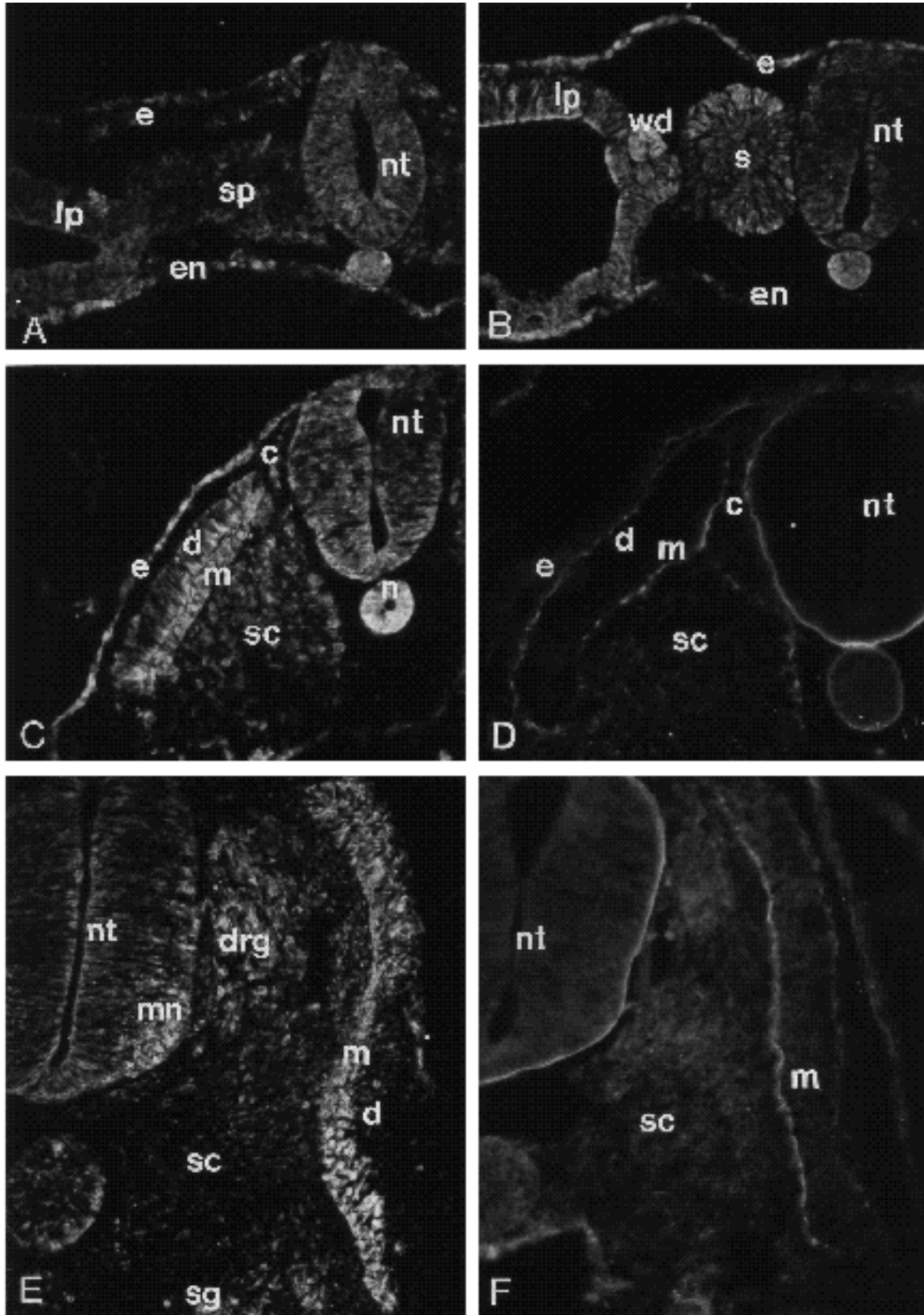
Fig. 2. 1 integrin expression during formation of the myotome. Transverse sections through (A) the segmental plate, (B) last somite and (C) seventh somite of a 25-somite stage embryo (E2.5) stained for 1 integrin. (D) Laminin staining of a transverse section of a 25-somite embryo at the same level as (C). (E, F) Transverse sections through the trunk of a 38-somite stage embryo (E3.5) stained for 1 integrin and laminin, respectively. 1 integrin staining is initially weak in the segmental plate and increases first during somite condensation. Staining becomes more pronounced after myotome formation and, later, is intense during myotome elongation at E3.5. Note that, at E2.5, all tissues including the neural tube, migrating neural crest, ectoderm, endodermal and lateral plate are faintly stained for 1 integrin. Only the notochord and the tip of the Wolffian duct exhibit stronger staining. At E3.5, in contrast, the sensory and sympathetic ganglion anlagen and the motoneurons in the spinal cord are stongly positive for 1 integrin. c, migrating neural crest cells; d, dermatome; drg, dorsal root ganglion; e, ectoderm; en, endoderm; lp, lateral plate; m, myotome; mn, motoneurons; n, notochord; nt, neural tube; s, somite; sc, sclerotome; sg, sympathetic ganglion; wd, Wolffian duct. (A-C, E, F, ×162; D, ×180).

Fig. 3. 1 integrin expression during ventral migration of myoblasts in the abdominal region and formation of abdominal muscles (level of the 25th-30th somite pairs). Transverse sections of embryos at E4.5 (A, B), E9.5 (C, D) and E14.5 (E, F). Staining for 1 (A, C, E) and laminin (B, D, F). Migrating myoblasts indicated by an arrow in A and B express high amounts of 1 integrin and are entirely surrounded by laminin. B is at a higher magnification than A in order to show the laminin pattern around individual migrating myoblasts. Note also that motor nerves are strongly labelled for 1 integrin (arrowhead). Early developing myotubes at E9.5 express adundant amounts of both 1 integrin and laminin, while mature myotubes at E14.5 are negative for 1 integrin, but retain laminin on their surfaces. Arrowheads in E and F point to capillary endothelial cells and smooth muscles around arteries which both show 1 integrin staining. kt, kidney tubules; m, myofibers; mt, myotome. (A, ×130; B, ×156; C, D, ×174; E, F, ×87).

extremely abundant along each myotube (Fig. 3D). After E11, myotubes became fully differentiated and contractile, and α1 integrin disappeared totally from their surfaces, whereas laminin was still retained in the cells' periphery (Fig. 3E, F). This disappearance of α1 integrin from skeletal muscles could also be evidenced by immunoblotting (Fig. 1E). The other skeletal muscles in the limb and head followed a similar pattern of α1 integrin expression, each with different developmental timing specificities. For example, facial muscles still expressed α1 integrin at stages when abdominal muscles had already lost their staining, probably reflecting differences in the timing of their maturation. After hatching, however, α1 integrin could not be detected in any skeletal muscle throughout the body.

α1 integrin expression in smooth muscles

Visceral smooth muscles

Visceral smooth muscle precursors appear gradually between E3 and E5 after local delamination of the splanchnic epithelium along the developing digestive tract. Even though the splanchnic epithelium was occasionally found to express α1 integrin prior to delamination, smooth muscle precursors were devoid of α1 integrin immunoreactivity at the time that they were released. This absence of α1 integrin expression is illustrated in the gizzard at E4 (Fig. 4A). Differentiation of visceral smooth muscle cells occurs sequentially along the digestive tract. It can be evidenced by the increase in the α-smooth muscle actin content of the cells, first rostrally in the esophagus (Fig. 4C), then progressively more caudally within the intestine, and finally in the cloaca and in the gizzard (Fig. 4E). The appearance of α1 integrin strictly followed the differentiation pattern of smooth muscle cells along the digestive tract. It could be detected first in developing smooth muscle cells in the esophagus by E5.5-E6 (Fig. 4B). α1 integrin staining was intense along the whole intestine by E7.5, and in the gizzard and cloaca by E9.5 (Fig. 4C). The conspicuous expression of α1 integrin in visceral smooth muscle was retained throughout development and was enhanced in adult animals compared to late embryos and newly hatched animals (Fig. 9). Immunoblotting experiments further demonstrated the increase in the α1 integrin content of the gizzard during development (Fig. 1D, E).

Pulmonary smooth muscles

α1 integrin was also expressed at high levels by smooth muscles in the pulmonary buds. Due to the relatively late development of the lung, α1 integrin was detectable in pulmonary smooth muscle cells only after E9.5 (Fig. 4F). Similar to what was observed with visceral smooth muscle, α1 integrin expression also increased in pulmonary smooth muscle after hatching (not shown).

Arterial smooth muscles

By E3 as the aorta becomes entirely surrounded by mesenchymal cells, a number of these cells clearly showed α1 integrin staining (Fig. 5A). Due to their high α-smooth muscle actin content, these cells could be unambiguously designated as early differentiating vascular smooth muscle cells (Fig. 5B). By E4.5, the dorsal aorta in the thoracic region was surrounded by several layers of cells which showed both strong α1 integrin and α-smooth muscle actin stainings (Fig. 5C, D). In contrast, in the most caudal regions, the dorsal aorta was less mature and showed very faint labeling for α1 integrin. Staining for α1 integrin on arterial smooth muscle cells did not change significantly until E14. By this stage, the number of smooth muscle layers around arteries again increased significantly, with a coincident enhancement of α1 integrin labeling (Fig. 9).

α1 integrin expression in the nervous system

Central nervous system

α1 integrin staining was not prominent in the neural tube during neurulation (Fig. 2A-C). However, the level of α1 integrin expression in the central nervous system changed at the time when neurons underwent morphological differentiation. The first neurons to exhibit strong α1 integrin staining could be detected in the ventral floor of the forebrain at the 20- to 25-somites stage (Fig. 6A). These cells were also intensely labelled for NC-1 (Fig. 6B) which has been shown previously to appear in early differentiating central neurons (Vincent and Thiery, 1984). High level of α1 integrin expression in the brain occurred between E3 and E5. As stratification of the brain began, staining was predominantly associated with the cellular layers (Fig. 6C). After E6, the α1 integrin content in the brain declined significantly and became almost undetectable by E9 (Fig. 6D).

The spinal cord followed essentially the same pattern of α1 integrin expression as the brain. Motor neuroblasts were the first cells to express high amounts of α1 integrin at the time when they extended neurites out into the sclerotome at E3-E3.5 (Fig. 7A). At this stage, staining was particularly strong on the cell bodies of the motoneurons. Later, as neurites started to regroup and form bundles Schwann cells, which were detected by NC-1 labeling, also exhibited strong α1 integrin staining (Fig. 7C). The high level of α1 integrin expression on Schwann cells could be correlated with the appearance of laminin along the developing nerve shaft (not shown, but see Rogers et al., 1986; Duband and Thiery, 1987). After E3.5-E4, α1 integrin expression was observed in most neurons within the spinal cord (Fig. 7B). The fibrous layers and the ependymal zone were less brightly stained than the neuron cell bodies. At our level of resolution, it was not possible to determine if glial cells were also stained. By E7.5, α1 integrin staining dropped to a relatively low level in the cellular layers (Fig. 7D) and, by E10, it was absent from the entire spinal cord (Fig. 7E). The disappearance of α1 integrin from the spinal cord could also be evidenced by immunoblotting (Fig. 1E).

Peripheral nervous system

In the trunk, the peripheral nervous system arises entirely from the neural crest. During migration, trunk neural crest cells were only weakly stained for α1 integrin (Fig. 2B, C). However, at E3 (stage-35 somite), α1 integrin expression was significantly enhanced in neural crest cells regrouping into the dorsal root ganglion anlagen (Figs 2E, 7A). This increase in staining was accompanied by the appearance of laminin and collagen IV within the crest cell population, which were first seen as dots, and subsequently as a thin basal lamina entirely surrounding the mass of the ganglion (not shown, but see Rogers et al., 1986; Duband and Thiery,
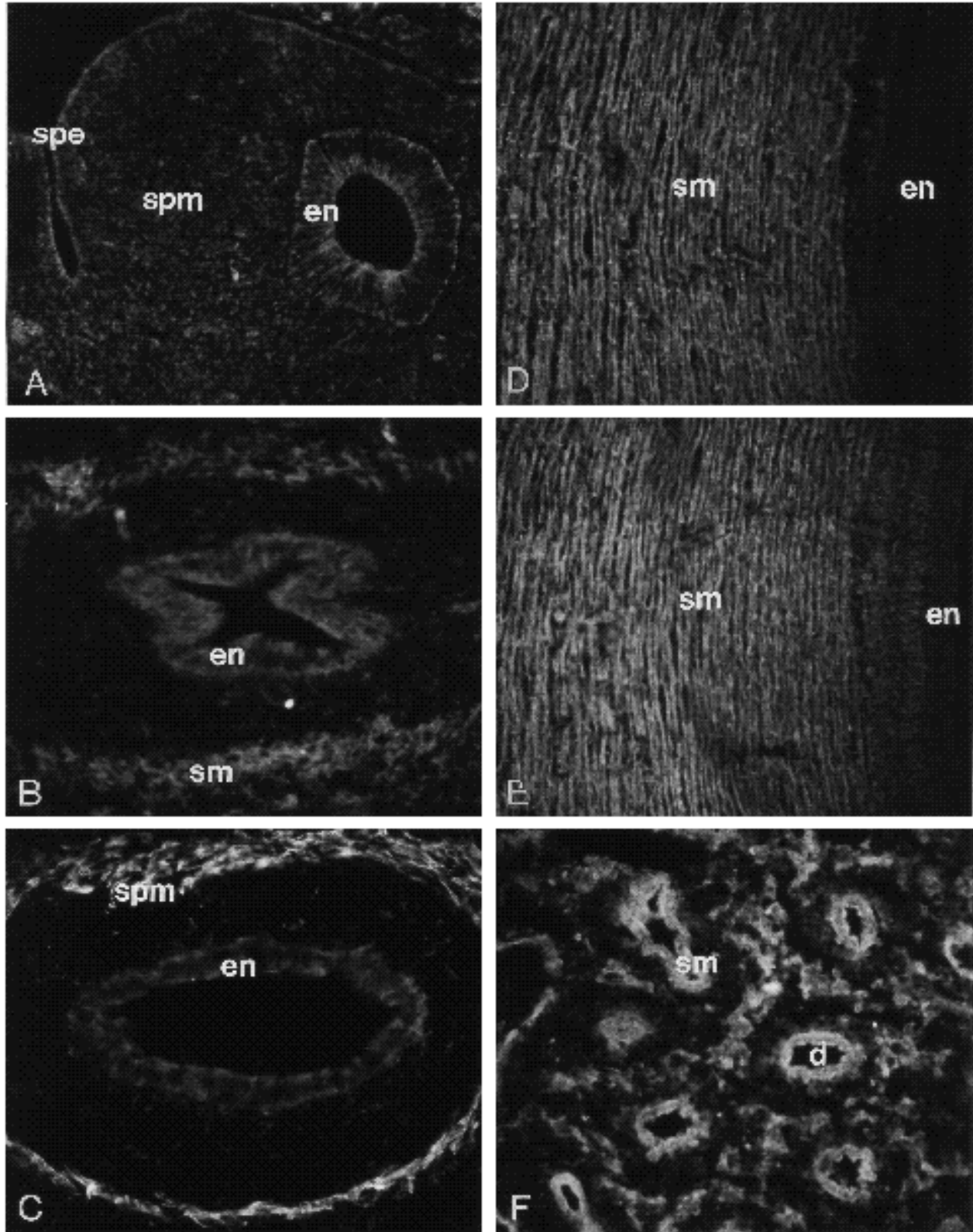
Fig. 4. 1 integrin expression during formation of visceral smooth muscles. Transverse sections through the gizzard at E4 (A) and E14.5 (D, E), the esophagus at E5.5 (B, C) and the pulmonary buds at E14.5 (F). (A, B, D, F) Stained for 1 integrin and (C, E) for -smooth muscle actin. 1 integrin is seen to appear gradually in visceral smooth muscles, first in the esophagus then progressively in the intestine and finally in the gizzard. Note that the endodermal epithelium expresses weak 1 integrin staining only in early stages. Pulmonary smooth muscles express intense 1 integrin staining. d, pulmonary duct; en, endoderm; sm, smooth muscle layers; spe, splanchnic epithelium; spm, splanchnic mesenchyme. (A, ×190; B, C, ×130; D, E, ×80; F, ×100).
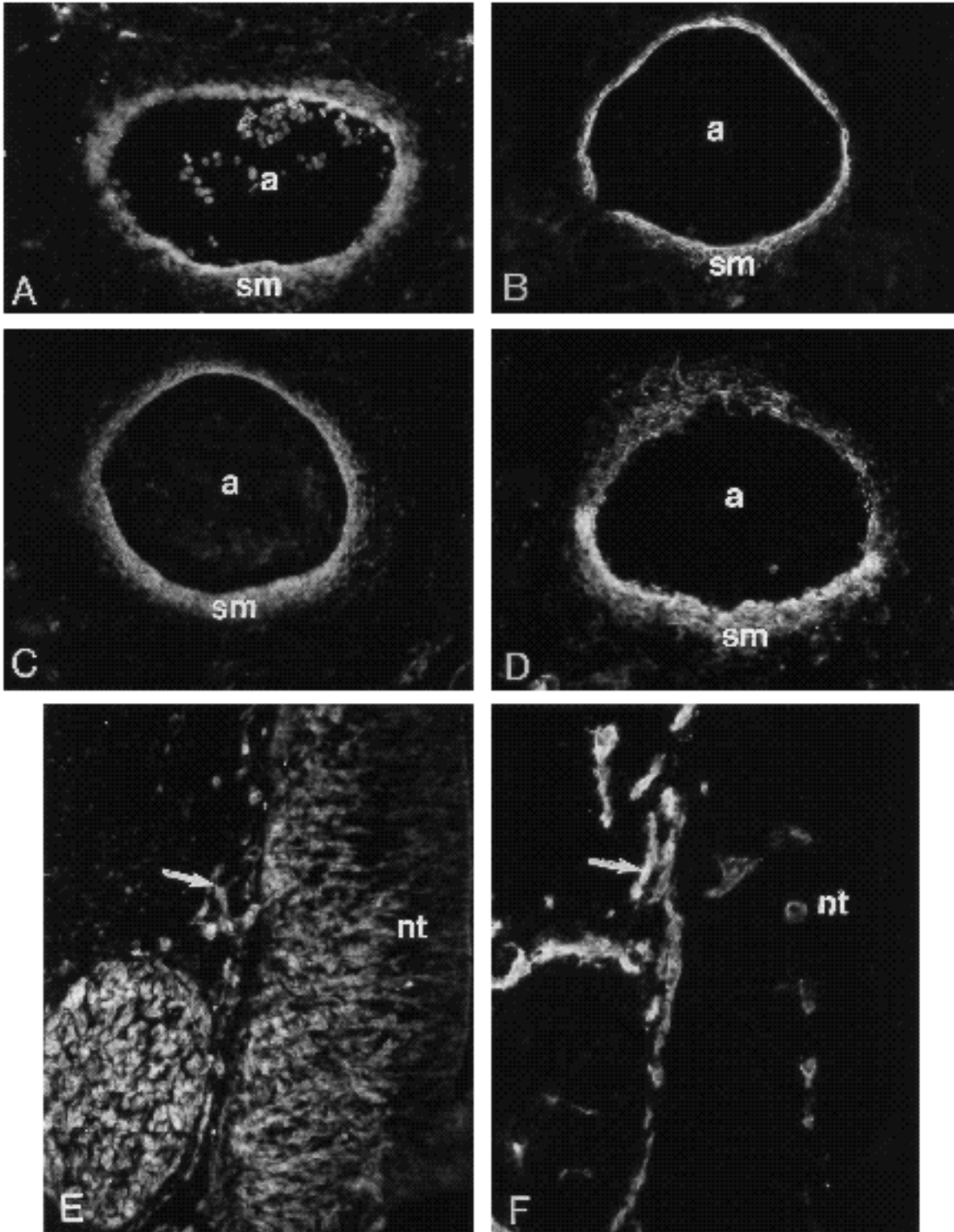
Fig. 5. 1 integrin expression during formation of arterial smooth muscles and in capillary endothelial cells. Transverse sections through the dorsal aorta at E3.5 (A, B) and E4.5 (C, D) and through the spinal cord at E4.5 (E, F). (A, C, E) staining for 1 integrin; (B, D) staining for -smooth muscle actin and (F) staining for MB-1, an endothelial cell marker. At E3-E3.5, a number of 1 integrin-positive mesenchymal cells regroup along the the aorta. These cells are also stained for for -smooth muscle actin. From E4 on, the aorta is surrounded by an increasing number of smooth muscle cell layers which express intense staining for 1 integrin. a, aorta; sm, smooth muscle layers; nt, neural tube. In E and F, endothelial cells (arrows) situated along the neural tube express strong 1 integrin. (A-D, ×100; E, F, ×180).
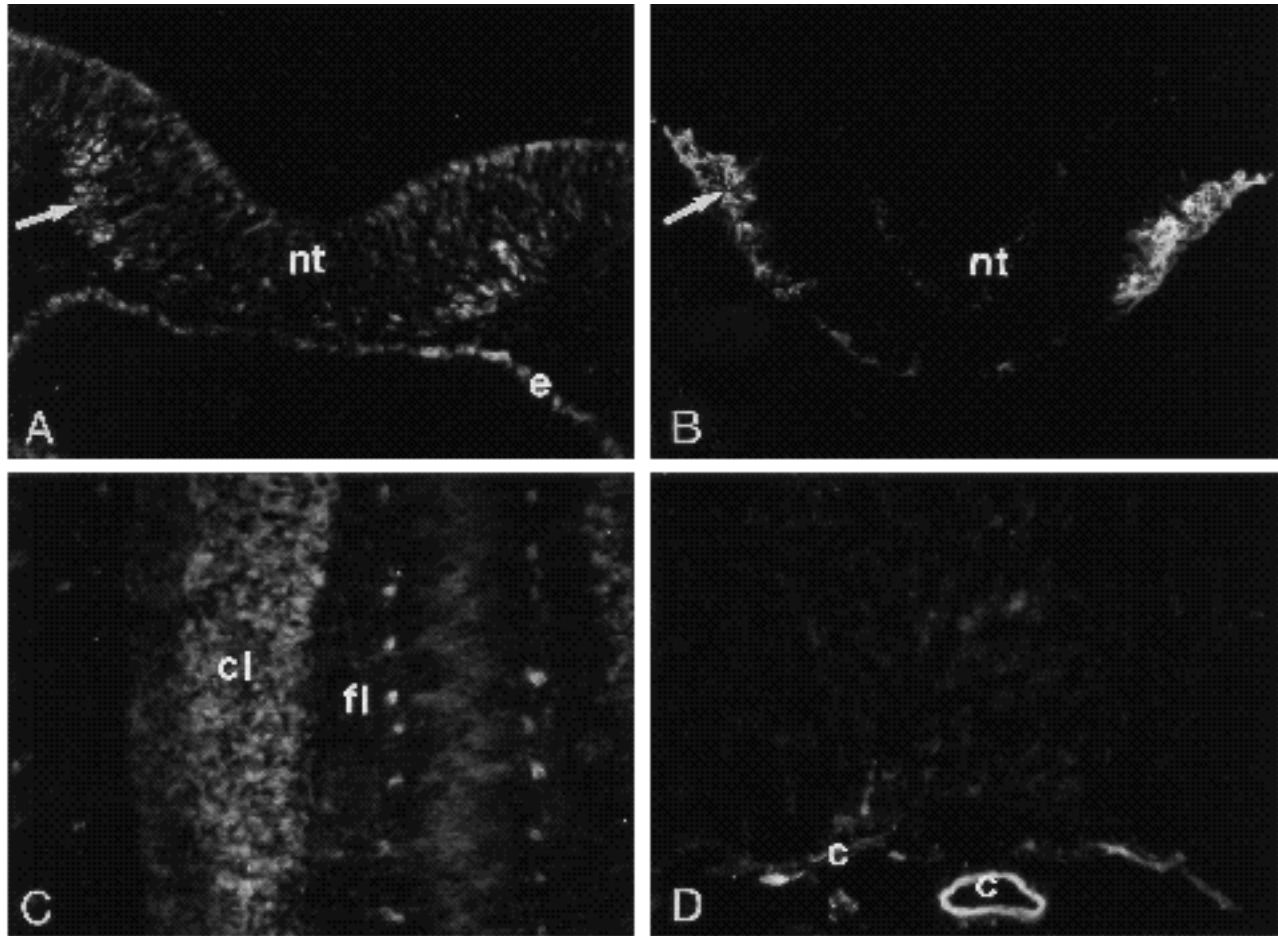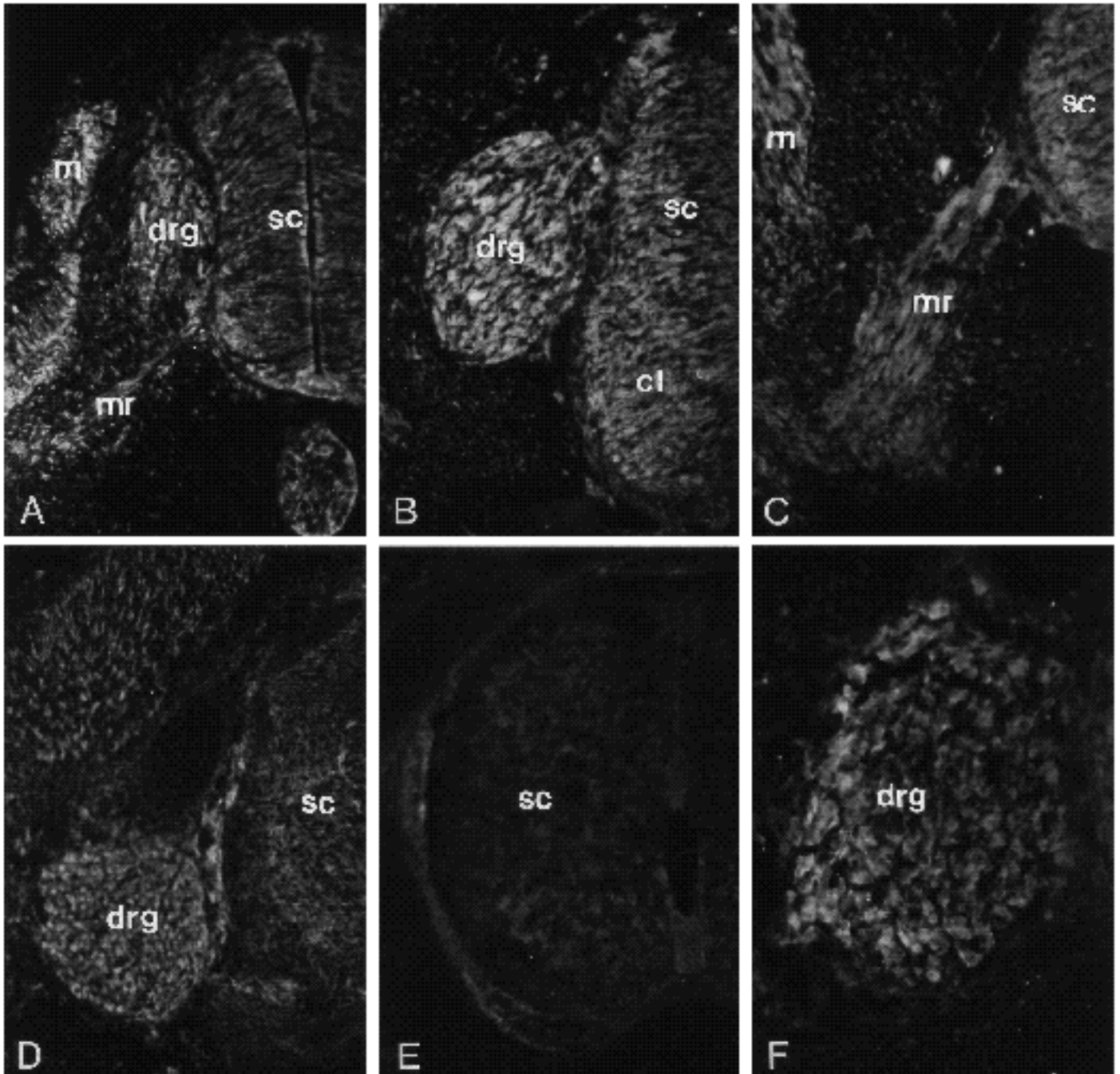
Fig. 6. 1 integrin expression in the brain. Transverse sections through the forebrain of a 25-somite stage embryo stained for 1 integrin (A) and the NC-1/HNK-1 epitope (B). Early differentiating neurons (arrows) identified by bright NC-1 staining express high amounts of 1 integrin and thus can be distinguished from neighboring cells. (C) Horizontal section through the hindbrain at E4.5 stained for 1 integrin and showing the heavily-stained cellular layer and the weakly stained fibrous layer. Cells brightly stained along the mid-line are capillaries as evidenced by -smooth muscle actin staining. (D) Transverse section through the brain at E9.5 stained for 1 integrin. No staining can be detected within the brain except in endothelial cells located at its periphery. c, capillaries; cl, cellular layer; e, ectoderm; fl, fibrous layer; nt, neural tube. (A, B, ×180; C, ×100; D, ×70).

1987). Expression of 1 integrin increased in the sensory spinal ganglia by E4-E4.5, as sensory neurons started to extend neurites (Fig. 7B). Spinal sensory ganglia retained strong 1 integrin staining for at least the following five days of development (Fig. 7D, F). During this period of time, laminin and collagen IV could be detected around individual neurons and along neurites (Duband and Thiery, 1987). After E11, 1 integrin staining declined significantly among the mass of the ganglion and was negative before hatching (not shown). 1 integrin expression also increased dramatically in the sympathetic ganglia at the time of their condensation along the aorta (Fig. 8A). However, in contrast to spinal ganglia, staining decreased rapidly to weak levels after E6 (not shown). It should be remembered that sympathetic ganglia express small amounts of laminin and collagens throughout development (Duband and Thiery, 1987).

The cranial peripheral ganglia also exhibited strong labeling for 1 integrin, each with developmental timing specificities. This strong 1 integrin staining could be generally correlated with the presence of laminin and collagen IV within the mass of the ganglion. For example, the trigeminal ganglion expressed high amounts of 1 integrin, laminin and collagen IV almost throughout embryonic development (Fig. 8C). Staining disappeared in the trigeminal ganglion only a few days before hatching. In contrast, the ciliary and the enteric ganglia, which expressed low levels of laminin and collagens, were stained for 1 integrin only transiently between E3.5 and E10 (Fig. 8B).

The ectodermal placodes at the origin of the sensory parts of the inner ear and of the olfactory organ expressed 1 integrin at high levels transiently, first during their formation, then during the extension of neurites from the placode towards the brain (Fig. 8D, E). The placodes lost their prominent 1 integrin staining later during development, possibly as neuronal connections were established. The anterior lobe of the hypophysis, which derives from a local invagination of the ectoderm (the Rathke's pouch) coming in contact with the floor of the forebrain, also exhibited a

Fig. 7. 1 integrin expression in the spinal cord and in the spinal sensory ganglia. Transverse sections through the brachial level at E3.5 (A), E4.5 (B, C), E7.5 (D) and E9.5 (E, F) stained for 1 integrin and showing the spinal cord, the motor root and the spinal sensory ganglia. In the spinal cord, staining appears first on the cell bodies of motoneurons, then expands to the whole cellular layer of the spinal cord and progressively disappears from it. Spinal ganglia, in contrast, express 1 integrin during the early regrouping of neural crest cells into the ganglion anlage and retain their staining for most of the embryonic development. Neurites issued from motoneurons are only faintly stained for 1 integrin, in contrast to Schwann cells which exhibit strong staining. cl, cellular layer of the spinal cord; drg, dorsal root (or spinal) ganglion; m, myotome; mr, motor root; sc, spinal cord. (A, ×180; B, C, ×130; D, ×90; E, ×110; F, ×150).

transient expression of 1 integrin during its formation (not shown).

Finally, the optic cup, which develops from the neural epithelium in the forebrain, was also found to express 1 integrin during its formation. Later, as the retinal ganglion cells became individualized and extended axons out of the retina into the brain approximately between E5 and E10 (Rager, 1980), they became brightly labelled for 1 integrin (Fig. 8F). Laminin was also found on the internal side of the retina where retinal ganglion cells are found and
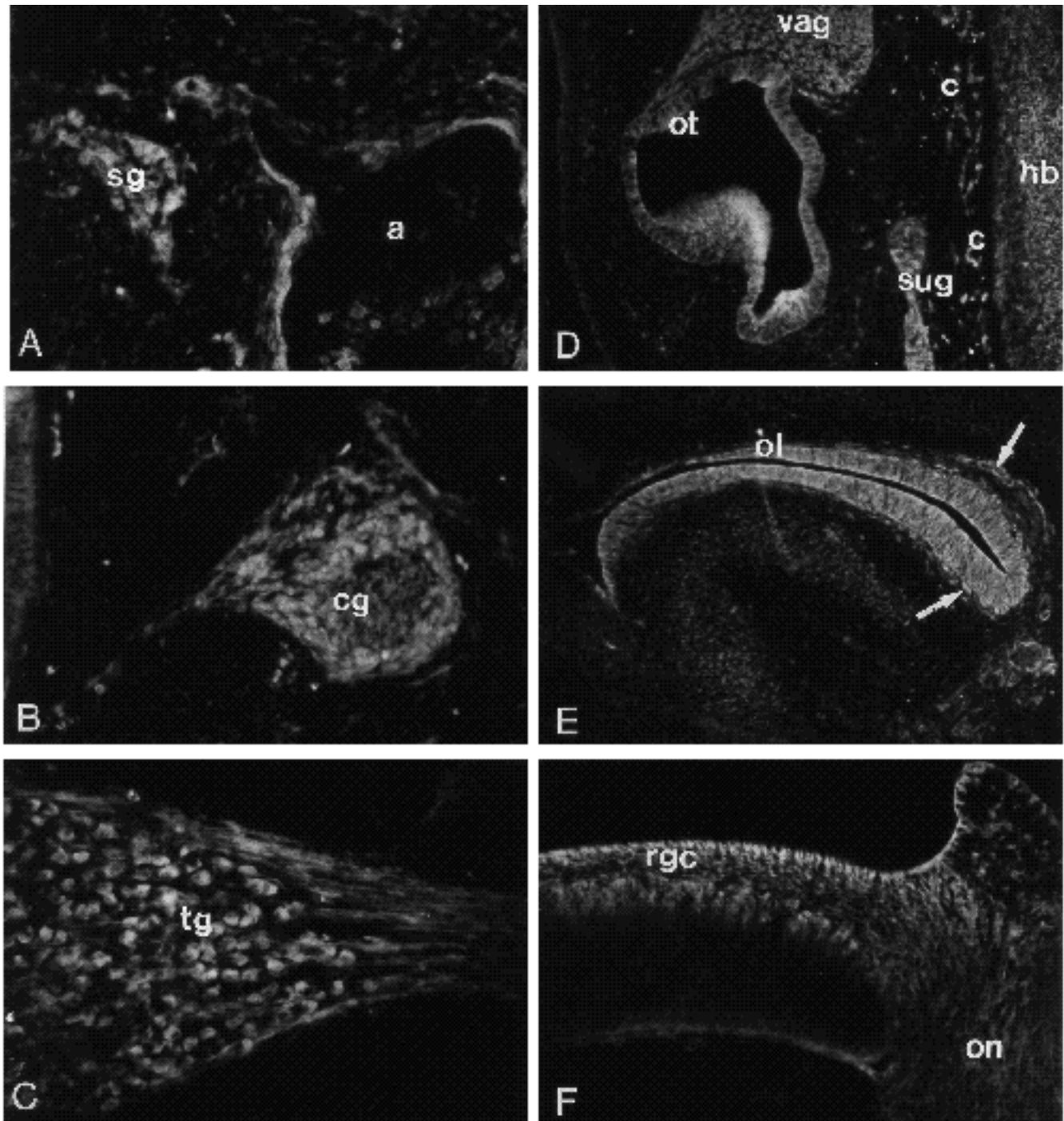
Fig. 8. 1 integrin expression in peripheral ganglia and in special sense organs. (A) Transverse section through the brachial level at E3.5 stained for 1 integrin showing the primordium of a sympathetic ganglion along the aorta. (B, C) Frontal section through the head at E9.5 stained for 1 integrin showing the ciliary and the trigeminal ganglia, respectively. Both ganglia exhibit strong 1 integrin staining. (D) Horizontal section through the hindbrain at E4.5 showing the heavily-labeled otic placode and vestibulo-accoustic ganglion. Note the numerous positively stained capillaries along the hindbrain and staining of the superior sensory ganglion. (E) Horizontal section through the forebrain at E7.5 showing the strongly stained epithelium of the olfactory placode and the nerve processes ending to it (arrows). (F) Horizontal section through the retina at E7.5 in the area of the optic nerve. Retinal ganglion cells and their neurite processes show strong
  1 integrin staining. a, aorta; c, capillaries; cg, ciliary ganglion; hb, hindbrain; ol, olfactory placode; on, optic nerve; ot, otic placode; rgc, retinal ganglion cells; sg, sympathetic ganglion; sug, superior ganglion; tg, trigeminal ganglion; vag, vestibulo-accoustic ganglion. (A, ×200; B, ×190; C, ×130; D, E, ×70; F, ×170).
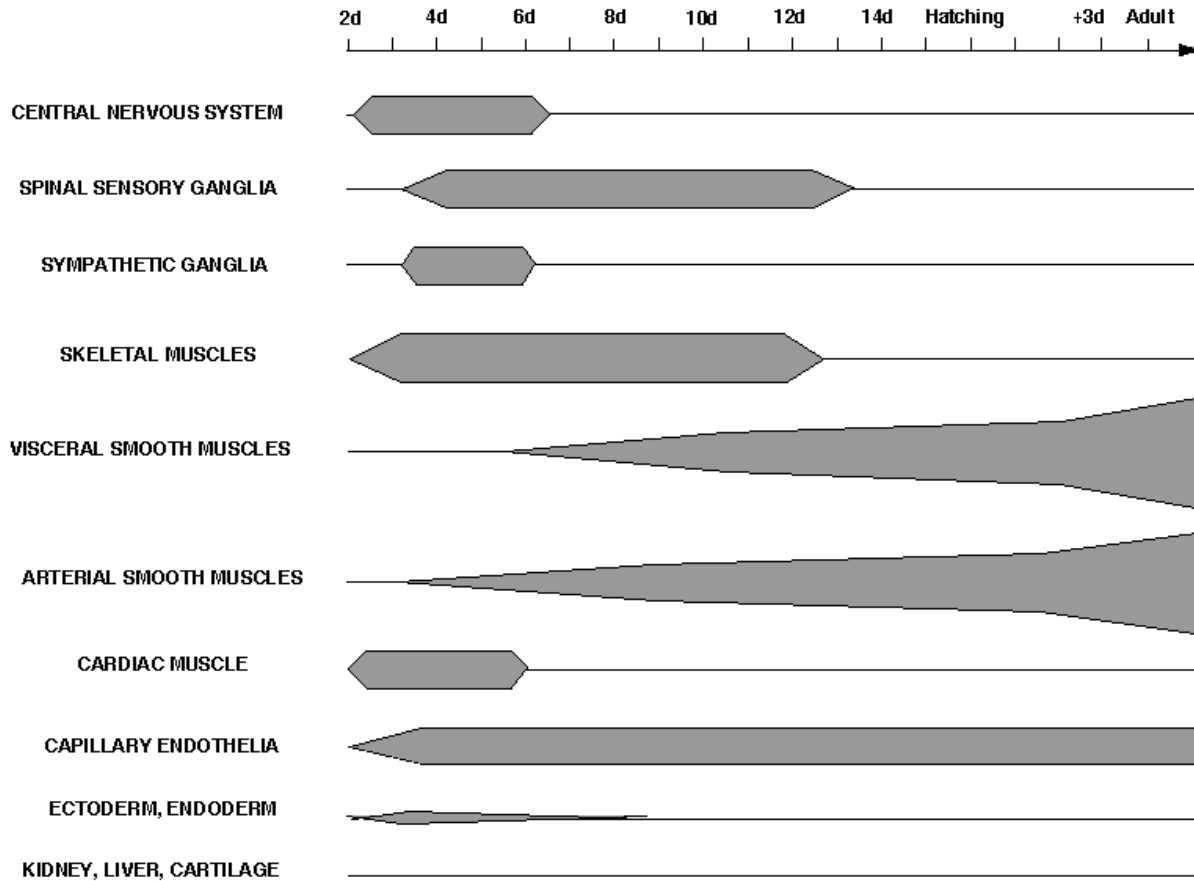
Fig. 9. Diagram summarizing the pattern of 1 integrin expression throughout development and in the adult avian animal.

along the optic nerve (not shown). After E12, however, both 1 integrin and laminin staining decreased among the retinal ganglion cells and along the optic nerve. This corresponded to the stage when virtually all retinal ganglion cells have reached the optic tectum (Rager, 1980).

α1 integrin expression in other tissues

Beside neural and muscular tissues, the only other cell types that expressed abundant amounts of 1 integrin were capillary endothelial cells and cardiomyocytes. 1 integrin immunoreactivity could be detected on capillary endothelial cells throughout development in a large variety of organs, including skeletal muscles (Fig. 3E), myocardium, kidney and dermis, and along the nervous system (Fig. 5E, F). 1 integrin was only transient in cardiomyocytes; staining was strong at the time of appearance and early differentiation of cardiomyocytes until E6 (Fig. 9). As described previously in the rat (Terracio et al., 1991), adult avian cardiomyocytes did not exhibit 1 integrin staining.

 1 integrin was also occasionally detected in a number of epithelial sheets. The ectoderm and the endoderm expressed varying amounts of 1 integrin only during the first half of the embryonic development. Transient 1 integrin expression in the human epidermis has also been reported recently (Hertle et al., 1991). In contrast, the liver, the pancreas, cartilages and most organs associated with the digestive tract never expressed 1 integrin. In the kidney,

all tubules were negative for 1 integrin. In this organ, beside capillary endothelial cells, the only cells that expressed 1 integrin at detectable levels were mesangial cells in the glomeruli, as also described previously in human kidney (Korhonen et al., 1990).

Discussion

It is commonly accepted that the components of the extracellular matrices and their cell surface integrin receptors play an important role in morphogenesis and cell differentiation. In this report, we have systematically investigated the expression of the 1 1 integrin during avian embryonic development and in the adult quail. We have found that (i) in adult avian tissues, 1 integrin was specifically expressed in visceral and vascular smooth muscles and in capillary endothelial cells, (ii) during development, 1 integrin showed a transient, dynamic distribution in striated muscles and in the nervous system and (iii) the expression of 1 integrin in muscles and nervous system was often correlated with the expression of laminin and collagen IV.

In this study, we used an antibody to a p175 membrane protein isolated from chicken gizzard smooth muscles (Koteliansky et al., 1985). The specificity of this antibody was compared with that of a recently characterized rabbit antiserum to avian 1 integrin (Syfrig et al., 1991), using

several different approaches. By immunoblotting of chicken gizzard extracts, both antibodies recognized a band of approximately 175×10^3 M_r and, by immunofluorescence staining of tissue sections, they gave identical patterns of labelling. In addition, the anti-p175 antibody recognized on immunoblots the β1 subunit of purified avian α1β1 integrin complex and immunoprecipitated the same proteins from human uterus smooth muscle extracts as a monoclonal antibody to human β1 integrin. Finally, when avian gizzard extracts were immunodepleted with the rabbit antiserum to avian β1 integrin or with the anti-p175 antibody, no bands comigrating with the β1 integrin were detected by immunoblotting. All together, these results strongly suggest that the anti-p175 protein antibody specifically recognized the β1 integrin subunit in both human and avian species.

α1β1 integrin has been shown to bind both laminin and collagens I, III and IV (Ignatius and Reichardt, 1988; Kramer and Marks, 1989; Turner et al., 1989; Belkin et al., 1990; Ignatius et al., 1990). More specifically, it interacts with the E1 domain of laminin and with a triple-helical region of collagen IV (Rossino et al., 1990; Sonnenberg et al., 1990b; Tomaselli et al., 1990; Vandenberg et al., 1991). A striking feature of the distribution of α1 integrin is that it is restricted to relatively few cell types both during development and in the adult (Fig. 9). With the exception of the early developmental stages, in which it is occasionally expressed at low levels in the ectoderm and endoderm, α1 integrin was almost never found in epithelial sheets. Moreover, it was found at low levels in tissues that adhere to connective-tissue-type collagens. One would have expected a laminin/collagen receptor to be expressed in a large number of tissues, most particularly in epithelia. It thus seems that α1 integrin may serve preferentially as a receptor for laminin and collagen IV in non-epithelial cells, such as the nervous system, striated and smooth muscle cells, and capillary endothelial cells. Our observation is consistent with a recent report showing that, in avian, collagen IV and to a lesser extent laminin are the preferential ligands of α1 integrin whereas collagen I does not bind this integrin (Syfrig et al., 1991). Therefore, one should assume that other cell types that are known to interact with laminin and collagen would utilize alternative integrin receptors. Other potential laminin and collagen receptors are the α2β1, α3β1, α6β1 and the α6β4 integrins. Connective tissue cells, including fibroblasts, are likely to adhere to collagens I and III through the α2β1 integrin (Elices and Hemler, 1989), whereas endothelial cells of large vessels are known to express α6β1 and α2β1 integrins as laminin receptors (Languino et al., 1989; Sonnenberg et al., 1990a). Among epithelial cells, adult human keratinocytes either in vitro or in vivo express α2β1, α3β1, α6β1 and α6β4 integrins as laminin/collagen receptors but not the α1β1 integrin (De Luca et al., 1990; Stepp et al., 1990; Hertle et al., 1991; Marchisio et al., 1991; Sonnenberg et al., 1991). Likewise, epithelial cells of the human nephric tubules exhibit the α2β1, α3β1 and α6β1 integrins (Korhonen et al., 1990). A complete survey of the distribution of the α6 and β4 integrin subunits in human adult tissues has been published recently (Sonnenberg et al., 1990a). It is of interest to note that most epithelial tissues were found to express α6 and

β4 integrins, with the noticeable exception of the liver, whereas most non-epithelial tissues that are known to interact with laminin and collagen IV (i.e. striated and smooth muscles, and nervous system) did not express these two integrins. These results, combined with our observations, would indicate that epithelial tissues would interact with laminin and collagen IV through one or a combination of the α6β1, α6β4, α2β1 and α3β1 integrins, while non-epithelial tissues adhere to the same extracellular matrix components using at least the α1β1 integrin. Such a difference in the expression pattern of laminin/collagen-binding integrins may possibly reflect functional specificities of the different members of the integrin family.

There are a number of possible explanations for the functions and developmentally regulated expression of β1 integrin during development and more specifically during myogenesis and neurogenesis. A possibility is that transient expression of the β1 integrin subunit reflects modifications in the composition of the extracellular matrix and, consequently, may correlate with changes in cellular behavior. This is probably the case for the nervous system. Our study reveals that sensory ganglia express high amounts of the β1 integrin subunit as long as they exhibit laminin and collagen IV. Conversely, α1β1 integrin expression is very transient in autonomic ganglia, correlating with a low laminin/collagen IV content. In the retina, both α1β1 and α6β1 integrins and laminin disappear from the surface of retinal ganglion cells after E11-E12, correlating with the fact that retinal neurons lose their ability to attach and extend neurites on laminin (this study; Cohen et al., 1986; de Curtis et al., 1991; Hall et al., 1987). Finally, retinoic acid and nerve growth factor, two naturally occurring morphogens, were found to increase β1 integrin expression in neuroblastoma cells in correlation with increased neurite response to laminin (Rossino et al., 1990, 1991). Thus, the patterns of expression of integrins and those of the extracellular matrix components are intimately interrelated. It has been found in in vitro studies that matrix components may control the surface distribution of integrins in cell-to-substratum anchorage sites (Dejana et al., 1988; Roman et al., 1989). It is then possible that, in vivo, integrin expression might also be regulated by extracellular matrix molecules during development of the nervous system.

The distribution of α1β1 integrin during neurogenesis at the time of nerve sprouting and on migrating myoblasts is consistent with a functional role of this integrin in cell locomotion over laminin and collagen IV substrata. In vivo, laminin and collagen IV have been detected in the nervous system at the time of neurite extension (Duband and Thiery, 1987; Hagg et al., 1989; Liesi, 1985; Rogers et al., 1986) and alongside migrating myoblasts (Solursh and Jensen, 1988; this study). In vitro, laminin has been shown to exert a key role in neurite sprouting for a large variety of neurons of either the central or peripheral nervous system (for reviews, see Reichardt and Tomaselli, 1991; Sanes, 1989) and to enhance motility of myoblasts (Öcalan et al., 1988). Likewise, collagen IV promotes neuritic outgrowth in various neurons (Lein et al., 1991). Integrin receptors, and more particularly the α1β1 integrin, are actively involved in cell adhesion to laminin and collagen IV during these processes of cell migration, as evidenced by in vitro and in

vivo perturbation experiments (Neff et al., 1982; Bozyczko and Horwitz, 1986; Tomaselli et al., 1986; Hall et al., 1987; Jaffredo et al., 1988; Ignatius and Reichardt, 1988; Turner et al., 1989; Rossino et al., 1990, 1991; Tawil et al., 1990; Tomaselli et al., 1990; Goodman et al., 1991; Lein et al., 1991).

Beside its possible role in cell migration during myogenesis and neurogenesis, 1 1 integrin may also be involved together with other 1 integrins in the differentiation program of these cells. Indeed, it has been shown that laminin promotes myogenesis in rat skeletal muscles in vitro (Foster et al., 1987) and that occupation of integrins by their corresponding ligand is a crucial event for several stages of myogenic differentiation (Menko and Boettiger, 1987). In *Drosophila*, the PS2 PS integrin is expressed in segmental muscle attachment sites and at Z-bands. This particular integrin is crucial in sarcomere formation, as integrin PS null myosopheroid mutants lack Z-bands and fail to form mature muscle fibers (Volk et al., 1990). The intense expression of 1 1 integrin in myoblast precursor cells coincides with the pattern of expression of *qmf1*, the avian homologue of *myf-5,* a member of the *MyoD* regulatory gene family (Braun et al., 1989; Charles de la Brousse and Emerson, 1990; Ott et al., 1991). This correlation in expression of a skeletal muscle master gene and of 1 1 integrin suggests that 1 1 integrin expression might be triggered by members of the *MyoD* family, which are responsible for switching on the differentiation program of myogenic precursors. Alternatively, 1 1 integrin itself may promote the expression of members of *MyoD* gene family, thus allowing initiation of myogenesis.

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