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The Id gene is activated by serum but is not required for de-differentiation in rat vascular smooth muscle cells

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Primary rat vascular smooth muscle cells cultured on fibronectin in the absence of serum lost smooth-muscle-specific myosin heavy chain but did not enter the cell cycle and proliferate until they were stimulated by serum. Under these conditions accumulation of Id mRNA occurred only in response to serum and was maximal during the G_1 phase of the cycle.

INTRODUCTION

Adult vascular smooth muscle cells (VSMCs) in the normal vessel wall express smooth-muscle-specific isoforms of myosin heavy chain (MHC) and actin. When VSMCs are dispersed in primary culture under appropriate conditions, the cells proliferate and the smooth-muscle-specific proteins are replaced by non-muscle isoforms [1–4]. It has been suggested that this phenotypic change is necessary before proliferation can occur [5,6] and this view is consistent with the observation that, at sites of pathological VSMC proliferation (e.g. in atheromatous plaques or after balloon-induced endothelial injury), VSMCs contain predominantly non-muscle proteins [1,7,8]. However, it has not been determined whether the loss of smooth-muscle-specific proteins and their replacement by non-muscle isoforms is obligatory for proliferation. To resolve this question, it will be necessary to block the change in phenotype specifically and determine whether the cells can proliferate while maintaining the full set of smooth-muscle-specific proteins. The genes which regulate the change in phenotype and the biochemical mechanisms which their products control are not known.

In skeletal muscle, the expression of muscle-specific proteins is controlled by a group of basic helix–loop–helix (HLH) proteins which induce transcription of the muscle-specific genes [9–11]. Recent studies have identified an HLH protein which lacks the basic DNA-binding region [12]. This protein, called Id, has been shown to form heterodimers with basic HLH proteins and prevent them binding to DNA.

Using a transient transfection assay, it was shown that overexpression of Id in C3H10T1/2 mouse embryo fibroblasts abolished the *trans* activation by MyoD of the muscle creatine kinase promoter linked to a chloramphenicol acetyltransferase reporter gene [12]. The Id gene may therefore inhibit differentiation and promote the de-differentiated state. However, studies on various types of proliferating and post-confluent non-proliferating cells have shown that Id expression may be down-regulated by withdrawal from the cell cycle. The data therefore leave open the possibility that Id expression is correlated with mitogenic stimulation and passage through the cell cycle rather than the promotion of de-differentiation.

We have recently reported that after plating freshly dispersed VSMCs on to fibronectin in the absence of serum, the cells remain viable but do not proliferate [13]. Nevertheless, they lose smooth-muscle-specific MHC at the same rate as cells which enter the cell cycle and proliferate in the presence of serum. Cells cultured under these two conditions have enabled the relationship between Id expression, stimulation by serum and de-differentiation, as marked by the loss of smooth-muscle-specific MHC, to be analysed.

MATERIALS AND METHODS

Cell culture

VSMCs were obtained by enzymic dispersal from adult Wistar rat aortae as previously described [13]. For cultures in serum, dispersed cells were suspended in medium 199 (Flow Laboratories) supplemented with glutamine (2 mM), penicillin (100 units/ml) and 10% foetal calf serum (FCS). Cells were plated at a density of 8×10^4 cells/cm^2. For serum-free cultures, the dispersed cells were suspended in the same medium without FCS and plated at the same density on to sterile bovine plasma fibronectin at 5 μg/cm^2 (Sigma). At no stage in this culture procedure were the aortae or the cells exposed to serum. Passaged cells were cultured in serum, passaged at a concentration of 1:2 (w/v) by trypsin treatment at confluence and used between passages 3 and 8. Cell counting was performed on trypsin-treated cells using a haemocytometer. The incorporation of [^{3}H]-thymidine into DNA and the relative amounts of the smooth-muscle-specific MHC isoform, SM-1, in samples containing the same number of cells were measured as previously described [13].

Northern analysis

Cytoplasmic RNA was isolated from cells cultured on 90 mm dishes. Briefly, the cells were harvested by trypsin treatment and washed once in phosphate-buffered saline (pH 7.4) containing NaCl (137 mM), Na_2HPO_4 (8.1 mM), KH_2PO_4 (1.5 mM) and KCl (2.7 mM) at room temperature. The cells were lysed in 0.4 ml of buffer containing NaCl (150 mM), Tris (10 mM), pH 7.4, $MgCl_2$ (1 mM) and Nonidet P40 (0.5%), and left for 3 min on ice. The samples were centrifuged (13000 *g*, 5 min) and the supernatants (0.4 ml) were extracted once with a solution containing 0.2 ml of phenol and 0.05 ml of SDS (10%), and once with 0.2 ml of phenol. The RNA was precipitated with sodium acetate (0.3 M) and 2.5 vol. of ethanol. The RNA samples (25 μg) were electrophoresed through 1.5% agarose/formaldehyde gels and transferred to Hybond N membranes (Amersham International). The RNA was hybridized with a random-primed ^{32}P-labelled Id

Abbreviations used: FCS, foetal calf serum; HLH, helix–loop–helix; MHC, myosin heavy chain; VSMC, vascular smooth muscle cell.
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cDNA probe [12] at 65 °C in 3 × SSC (1 × SSC contains 150 mM-NaCl and 15 mM-sodium citrate), dextran sulphate (5%), 10 × Denhardt's solution, salmon sperm DNA (250 μg/ml) and SDS (0.1%) overnight. The blots were washed twice at 65 °C for 30 min in 0.1 × SSC/SDS (0.1%) before exposure to Fuji RX X-ray film.

RESULTS AND DISCUSSION

VSMCs cultured in the presence of serum doubled in number after about 72 h and underwent exponential growth for the next 72 h, before reaching a stationary phase at constant cell number after 144 h (Fig. 1). Over the first 72 h of culture in serum the cells lost approx. 70% of the total smooth muscle MHC before the first division was complete, and after 144 h the cells contained only 5% of the original smooth muscle myosin per cell compared with the freshly dispersed VSMCs (Fig. 2*a*). However, after the cells had reached the stationary phase, there was a re-accumulation of smooth muscle MHC by 192 h to approx. 20% of the initial amount per cell at 0 h. If serum was withdrawn from the cells at 144 h, the amount of smooth muscle MHC increased further, to 40% of the amount initially present in the cells (Fig. 2*b*). These data show that, as defined by the expression of smooth muscle MHC, there is a partial re-differentiation in the stationary phase which is enhanced by the removal of serum.

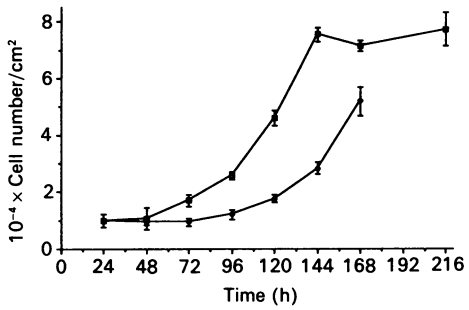

Fig. 1. Time course of proliferation of primary VSMCs

Cells were plated (8×10^4/cm^2) at $t = 0$ in the presence of serum (⊡) or on to fibronectin in the absence of serum for 72 h before serum was added (◆), as described in the Materials and methods section. Values for cell numbers are the means of five separate experiments analysed in triplicate; error bars are S.D. values.

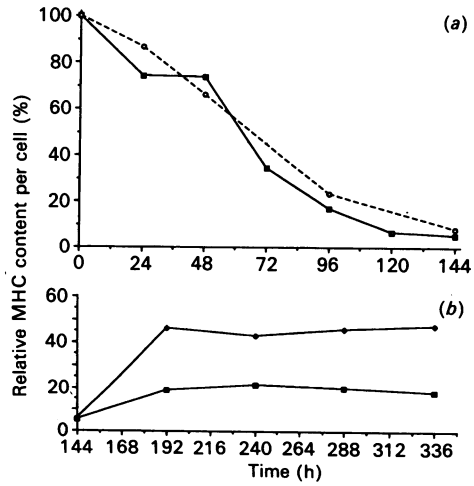

Fig. 2. Smooth muscle MHC in primary VSMCs in the presence and absence of serum

(*a*) Relative amounts of smooth muscle MHC per VSMC over the first 144 h of culture in the presence of serum (⊡) or cultured in the absence of serum on fibronectin (○). (*b*) Re-appearance of smooth muscle MHC during the stationary phase in cells cultured continuously in the presence of serum (⊡) or after serum withdrawal at 144 h (◆).

If the cells were cultured on fibronectin in the absence of serum, their number remained constant, and when serum was added at 72 h the cells had doubled in number after a further 48 h (Fig. 1). Under these conditions, the rate of loss of smooth muscle MHC was very similar to that in the cells cultured in the presence of serum from 0 h (Fig. 2*a*). This shows that the loss of smooth mucle MHC is not dependent on mitogenic stimulation by serum and occurs independently of entry into the cell cycle.

To determine whether Id expression in the primary VSMCs is linked to de-differentiation, as indicated by the loss of smooth muscle MHC, or to stimulation of the cells by serum, the amounts of Id mRNA were compared under the two sets of culture conditions described above. Northern blot analysis for Id expression was performed on RNA extracted from cells cultured in the presence of serum in exponential growth at 120 h, in stationary phase at 192 h and from cells withdrawn from serum for 24 h after 120 h and 192 h. There was a substantial amount of Id mRNA in the cells at both 120 h and 192 h, but this was markedly decreased 24 h after the withdrawal of serum at each time (Fig. 3). It should be noted that in all experiments Id was detected as a doublet, presumably corresponding to two transcripts of the gene [14].

These data demonstrate that the expression of large amounts of Id mRNA is correlated with the presence of serum whether the cells are growing exponentially (i.e. are in the cell cycle) or are in the stationary phase. Removal of serum from the cells in either state results in the down-regulation of Id mRNA. It is also clear that Id expression can occur both when the amount of smooth muscle MHC in the cells has been substantially decreased in the exponentially growing cells at 120 h and when the cells are re-expressing smooth muscle MHC in the stationary phase at 192 h.

Further evidence consistent with these conclusions was obtained from cells cultured on fibronectin in the absence of serum. Very low amounts of Id were detected in RNA obtained from cells at 1 h and 5 h after dispersal and the amount of Id in the cells cultured on fibronectin in the absence of serum after 72 h was below the level of detection. These data indicate that a large early increase in the expression of Id does not occur in response to dispersal of the cells and that expression of the Id gene is not correlated with the loss of smooth muscle MHC in the serum-free cultures.

Experiments were then performed to determine the time course of Id expression in response to serum. Cells were cultured on fibronectin for 48 h before the addition of serum and RNA was extracted from cell samples at various times afterwards. The amount of Id RNA increased over the first 9 h after serum stimulation and declined substantially between 24 h and 48 h (Fig. 4*a*). The time course of [^{3}H]thymidine incorporation into replicates of the cell samples showed that the cells started to enter S phase at 20 h after serum addition, demonstrating that Id expression was activated early in G_1 under these conditions.

Similar experiments were also carried out on the effect of serum on Id expression in passaged cells. The amount of smooth muscle MHC in passaged cells is very low compared with that in freshly dispersed cells and the smooth muscle MHC is not significantly re-expressed when the passaged cells reach stationary phase (D. J. Grainger, unpublished work). Any changes in Id

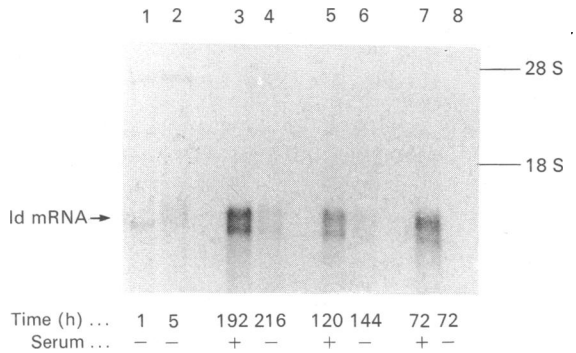

Fig. 3. Id expression in cultured VSMCs

Lane 1, VSMCs were dispersed and maintained in the absence of serum and RNA was extracted after 1 h. Lane 2, VSMCs were dispersed and maintained in the absence of serum and RNA was extracted after 5 h. Lane 3, VSMCs were cultured in the presence of serum and RNA was extracted after 192 h. Lane 4, VSMCs were cultured in the presence of serum for 192 h and serum was withdrawn for 24 h prior to RNA extraction at 216 h. Lane 5, VSMCs were cultured in the presence of serum and RNA was extracted after 120 h. Lane 6, VSMCs were cultured in the presence of serum for 120 h and serum was withdrawn for 24 h prior to RNA extraction at 144 h. Lane 7, VSMCs were cultured in the absence of serum on fibronectin for 48 h and serum was added for 24 h prior to RNA extraction at 72 h. Lane 8, VSMCs were cultured in the absence of serum on fibronectin and RNA was extracted after 72 h. Blots were exposed to X-ray film for 4 days at −70 °C under one intensifying screen. The positions of the 28 S and 18 S rRNAs are indicated.

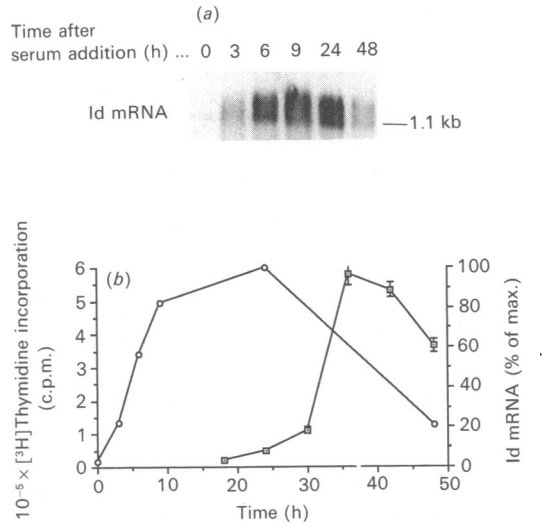

Fig. 4. Time course of Id expression and DNA synthesis in primary VSMCs after serum addition

(*a*) VSMCs were cultured in the absence of serum on fibronectin for 48 h. Serum was added and RNA was extracted from the cells after 0, 3, 6, 9, 24 and 48 h. (*b*) DNA synthesis and Id expression in primary VSMCs. The incorporation of [^{3}H]thymidine (2 mCi/ml) was assayed after incubation for 2 h (⊡). Relative amount of Id mRNA, normalized to the maximal response, were measured by densitometric scanning of the lanes in (*a*), normalized for RNA loading in each lane (○). Blots were exposed as described for Fig. 3.

expression in passaged cells cannot, therefore, be correlated with smooth muscle MHC. Exponentially growing and stationary passaged cells both expressed Id in the presence of serum and withdrawal of serum for 24 h from cells in either state resulted in

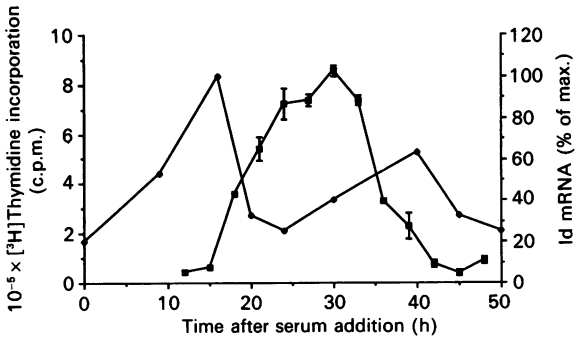

Fig. 5. Time course of Id expression and DNA synthesis in passaged VSMCs after serum addition

Serum was withdrawn for 48 h from exponentially growing passaged VSMCs. DNA synthesis (⊡) and relative amounts of Id mRNA (◆) were measured at various times after the re-addition of serum.

a large decrease in the amount of Id, similar to that observed in primary VSMCs (see Fig. 3). The time course of Id expression and [^{3}H]thymidine incorporation into DNA in passaged cells after the re-addition of serum to cells that had been deprived of serum for 48 h is shown in Fig. 5. The experiments show that a large increase in Id expression occurred early in G_1 in response to serum in the passaged cells, with a time course similar to that of the primary cells. However, entry into S phase and the decline in Id expression both occurred earlier in the passaged cells than in the primary VSMCs. These observations are therefore consistent with regulation of the Id gene being coupled to the cell cycle.

In conclusion, the data are consistent with regulation of the Id gene by the presence of serum and with the recent suggestion, in a review by Weintraub *et al.* [9], that Id may be under the control of a serum response factor. By contrast, there is no simple correlation between the expression of smooth muscle MHC and Id expression, suggesting that the Id gene is not involved in the de-differentiation of the VSMCs defined by this protein. Any role for the Id gene in regulating the cell cycle remains to be determined.

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