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Effect of Laminin on the Nuclear Localization of Nucleolin in Rat Intestinal Epithelial IEC-6 Cells

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Laminin is a major component of extracellular matrix. The mechanism of action of laminin on cell proliferation, differentiation, and migration is not fully understood. In this study, we investigated the role of extracellular matrix, especially laminin, on the cellular localization of the nuclear protein, nucleolin, and on cell proliferation. Immunofluorescent and western blot analysis indicated that nucleolin was translocated most efficiently to the nucleus in the small intestinal rat epithelial cell line (IEC-6) when cultured on laminin-coated plates. Specifically, nucleolin was observed predominantly in cytoplasm in the cells cultured without laminin. In contrast, nuclear localization was observed in the cells cultured on laminin. This effect of laminin on nucleolin translocation was time-dependent. Laminin was also observed to stimulate proliferation of IEC-6 cells in serum free medium. Our results suggest that laminin alters the distribution of nucleolin which may be an early signal for cell proliferation.

Nucleolin is an abundant nuclear phosphoprotein found in eukaryotic cells and is a principle protein constituent of the nucleolar organizing region (NOR) (1–4). Several studies have indicated nucleolin has an essential role in regulating rDNA transcription (5,6), packaging of nascent ribosomal RNA (2–7), and maturation of rRNA (7,8). While the exact function of nucleolin on cell proliferation is unclear, it is known that the amount of cellular NOR reflects ribosomal biosynthesis levels which is an indicator of cell proliferation (9).

Nucleolin has been demonstrated to shuttle between the nucleus and cytoplasm independently of the cell cycle (10-12). Moreover, recent reports utilizing phosphoprotein analysis have suggested that nucleolin may also be localized to the surface membrane in various cells (13,14). The function of cytoplasmic and membrane-associated nucleolin remains to be established. However, membrane-associated nucleolin has been shown to act as a receptor for enterovirus (14), suggesting that it may have a receptor function.

Extracellular matrix (ECM) play an important role in regulating the proliferation, differentiation and migration of gastrointestinal epithelial cells (15-17). Laminin, a glycoprotein consisting of three polypeptide chains is a major bioactive constituent of ECM (15,17). The effects of laminin on cell proliferation, differentiation and migration have been demonstrated in a number of cell types including intestinal epithelial cells (16,18-20). Several biologically active motifs have been identified in the three chains of laminin (21-23). A protein binding region in the C-terminus of the long arm of laminin that consisting of amino acid sequence ile-lys-val-ala-val (IKVAV), has recently been characterized by Kibbey *et al.* (24,25). This motif appears to represent a binding site for several proteins including nucleolin (24,25). At present, the functional relationship between laminin and nucleolin in unknown. However, these findings raise the possibility that an interaction between laminin and nucleolin may represent a novel signaling mechanism which modifies the cellular response of intestinal epithelial cells.

In this study, we investigated the effect of laminin and several other ECM components on the cellular distribution of nucleolin in a rat intestinal epithelial cell line and on cell proliferation. It was observed that the translocation of nucleolin from the cytoplasm to the nucleus was significantly enhanced following exposure of epithelial cells to laminin. This translocation preceded laminin dependent cell proliferation suggesting a functional link between nucleolin distribution and cell proliferation.

Abbreviations used: ATCC, American Type Culture Collection; DMEM, Dubecco's modified eagles medium; ECM, extracellular matrix; FBS, fetal bovine serum; HRP, horse radish peroxidase; NOR, nuclear organizing region; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride.

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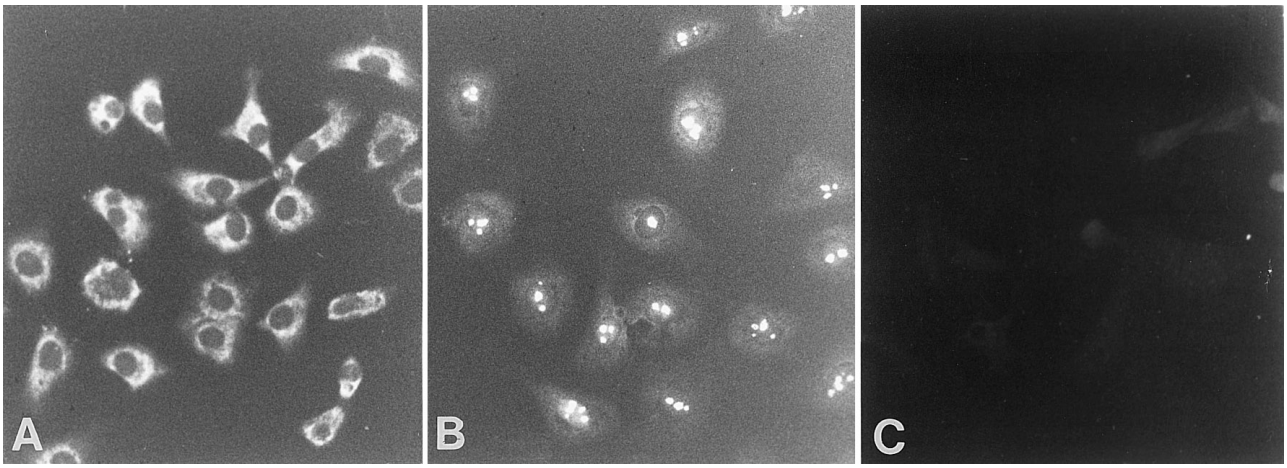

FIG. 1. Immunofluorescent staining of nucleolin. IEC-6 cells were plated on laminin-coated (Panel B) and uncoated (Panel A) coverslip, and cultured for 15 hr Immunofluorescent staining of nucleolin was carried out as described in Materials and Methods. Panel C is a control experiment utilizing the preimmune serum for staining.

MATERIALS AND METHODS

Cell culture. IEC-6 cell line were obtained from ATCC (Rockville, MD) and were cultured in DMEM with 5% FBS and supplemented with insulin (0.1 units/ml). Glass coverslips were coated with laminin (20 μg/ml) (a gift from Dr. P. Liesi, NIAAA, Rockville, MD), rat fibronectin (20 μg/ml) (Sigma, MO) Collagen IV (20 μg/ml) (a gift from D. H. K. Kleinman, NIDR, Bethesda, MD) and poly-d-lysine (20 μg/ml) (Sigma, MO) and allowed to sit at 4°C for 18 hr. Cells were seeded at 1.5×10^4 cells/well on coated or uncoated glass coverslips in 6-well tissue culture plates. Cell number were determined on a daily basis using a hemocytometer to determine the rate of cell proliferation.

Immunofluorescence analysis. Cells were plated on the glass coverslip at a density of 1.5×10^4 cells/well and cultured for up to 15 hr. Cells were washed with PBS and fixed in methanol at −20°C for 5 min. For nonpermeabilized experiments, cells were fixed with PBS containing 4% formaldehyde for 30 min at 37°C. The samples were blocked with PBS containing 3% BSA for 30 min at 37°C. After removing the blocking solution, the samples were incubated for 1 hr at room temperature with anti-nucleolin polyclonal antibody produced against purified nucleolin extracted from 3T3-F442A cells (1:2000) (26). In some experiments, a rabbit polyclonal anti-serum (1:2000) raised against an unrelated but highly conserved 62 KDa protein, designated as pErR (Slomiany, Woldehawariat and Petryshyn, manuscript in preparation) was used to examine the specificity of the laminin effect. Following washings with PBS/1% Triton X-100, biotin conjugated goat anti-rabbit secondary antibody (1:1000) (Sigma, MO) was applied for 1 hr at room temperature. The samples were washed extensively three times and incubated with streptavidin-FITC (1:100) Kirkegaard & Perry Laboratory, MD) for 30 min at 37°C. The samples were washed again, then wet-mounted with 1:1 PBS:glycerol. Epifluorescence was visualized and photographed using a Nikon HFX-II microscope.

Preparation of cell fractions. Briefly, cells were cultured on laminin coated plates and non-coated plates at 1×10^7/plate. After 15 hr of culture, cells were washed twice with ice-cold PBS and scraped in 5 ml ice-cold PBS. Cells were collected by centrifugation at 600 × g for 5 min at 4°C. Cells were disrupted in lysis buffer containing 10 mM Tris-HCl (pH 7.7), 80 mM KCl, 6 mM $MgCl_2$, 2 mM dithiothreitol, 250 mM sucrose, and 0.1% Triton X-100 for 10 min on ice (26). The homogenates were centrifuged at 10,000 × g for 10 min at 4°C. The crude cytoplasm supernatant (S10) were removed and stored at −70°C for later use. The pellet (P10) were re-suspended in 100 μl of nuclei lysis buffer A (12 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.0), 0.88 M sucrose, 1 mM PMSF and 5 μg/ml leupeptin) (26). The suspension was sonicated 60 s to disrupt the nuclei and subjected to centrifugation 20 min at 1100 × g at 4°C. The supernatant (S1) was saved and the pellet re-suspended in 100 μl of nuclei lysis buffer B (1 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.0), 0.88 M sucrose, 1 mM PMSF and 5 μg/ml leupeptin) as previously described (26).

Immunoblotting analysis. To examine the distribution of nucleolin between nuclear and cytoplasmic compartments, western blotting analysis was performed. Extracts from equal numbers of cells (1×10^6) were separated at 7% SDS-PAGE. The proteins were then transferred to PVDF membrane in a semi-dry blotting apparatus (Bio-Rad, CA). The membrane was washed twice with PBS and then incubated with 5% dry nonfat milk in PBS for 1 hr at room temperature. After three 5 min washes at room temperature with PBS-T (0.1% Triton X-100 in PBS), the membrane was incubated with anti-nucleolin antiserum or pre-immune serum (1:1000) as a control for 1 hr at room temperature. Following three 5 min washings at 25°C, the membrane was incubated with goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (1:10,000) (Sigma, MO). Following three washings, HRP activity was detected by enhanced chemiluminescence (Pierce, IL) according to manufacturer's instructions and exposed to a Kodak BioMaxi X-ray film (Kodak, CT).

RESULTS

Effect of Laminin on the Localization of Nucleolin

The effect of laminin on the distribution of nucleolin was examined immunologically in IEC-6 cells plated on laminin-coated and uncoated coverslips using antiserum previously shown to be highly specific for nucleolin (24,26). Following 15 hr of incubation, an intensive immunofluorescent staining of nucleolin was observed in the cytoplasm of cells cultured on uncoated glass coverslips. However, little or no staining of nucleolin was detected in the nucleus under these conditions (Fig. 1A). The clear, round appearance of the nucleus indicates that the cells were not undergoing mitosis. In contrasts, on laminin-coated coverslip nucleolin was

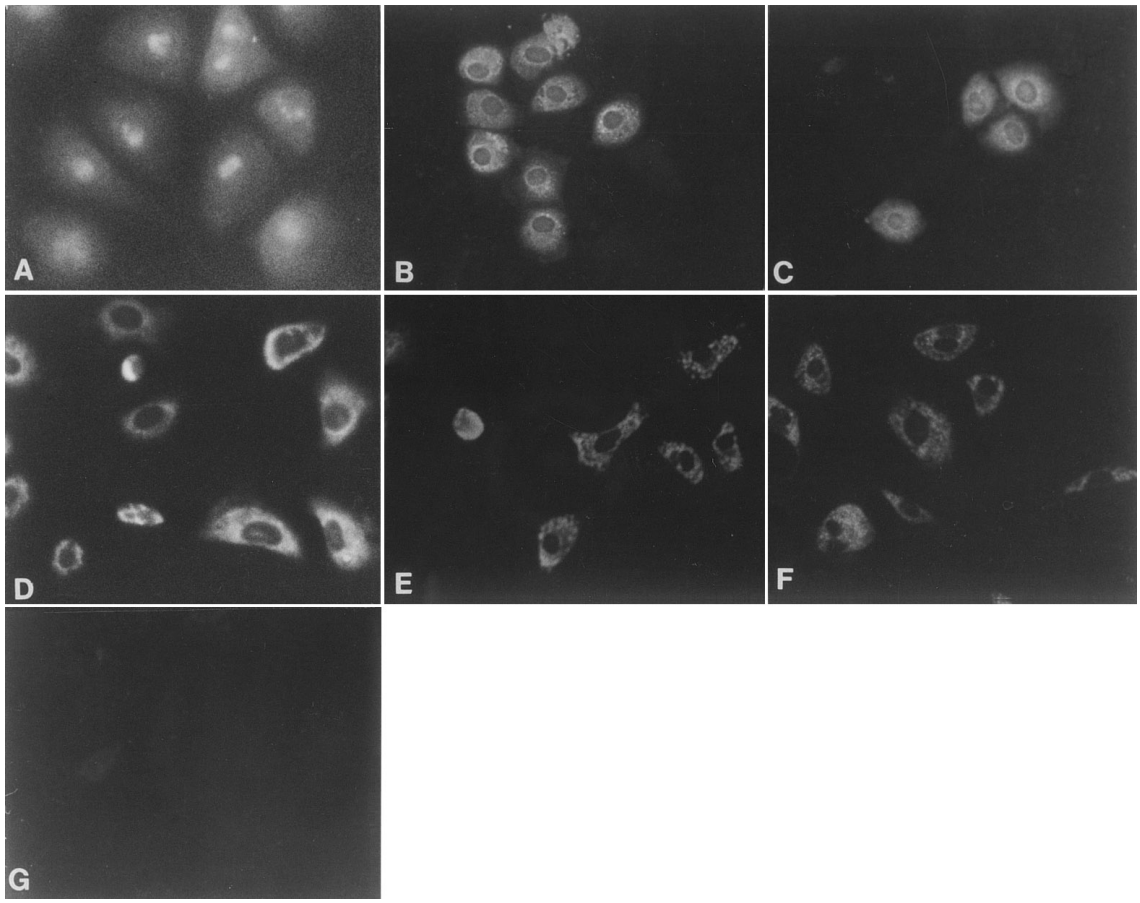

FIG. 2. Immunofluorescent staining of nucleolin in IEC-6 cells cultured on laminin and other substratum. Cell culture and immunofluorescent staining with antinucleolin and anti-pErR sera were as described in figure 1. Culture plates were treated as follows: Panels A, F and G, laminin coated; Panel B, fibronectin coated; Panel C, collagen IV coated; Panel D, poly-D-lysine; Panel E, no coating. Immunofluorescent staining of pErR IEC-6 cells were cultured on laminin coated (Panel F) and uncoated (Panel E) coverslips. The immunostaining of the preimmune serum for pErR is also shown (panel G).

predominantly localized to the nucleoli of the nucleus and markedly reduced in the cytoplasm (Fig. 1B). The immunostaining observed was specific for nucleolin because the pre-immune serum obtained from the same animal used to raise the anti-nucleolin antibody, showed no positive staining either in the presence of (Fig. 1C) or absence of laminin (data not shown). These results suggest the laminin markedly influences the cellular distribution and localization of nucleolin in IEC-6 cells.

To examine both the specificity of the laminin effect on nucleolin translocation and its dependence on adhesion of cells to biological substratum, IEC-6 cells were plated on coverslips individually coated with two other major ECM components, fibronectin and collagen IV, and with the adhesion facilitator poly-D-lysine. As indicated in Fig. 2 B-D, after 15 hr of culture, on plates coated with fibronectin, collagen IV and poly-D-lysine, nucleolin remained extensively localized to the cytoplasm of cells. The pattern of staining was similar to that observed in the absence of laminin (Fig. 1A) except for collagen IV, where a small amount of nucleolin was consistently localized to the nucleus (Fig. 2C). In contrast, cells cultured on laminin-coated plates exhibited a marked localization of nucleolin to the nucleoli of the nucleus (Fig. 2A). The absence or reduction of nuclear staining in the absence of laminin is unlikely to result from a lack of adhesion of cells to uncoated coverslips because both fibronectin and collagen IV serve as substratum for adhesion. Moreover, plating on a poly-D-lysine substratum to facilitate adhesion was also ineffective in localizing nucleolin to the nuclear compartment (Fig. 2D). These observations suggest that nuclear localization of nucleolin is significantly enhanced by a laminin-dependent process. To explore this further, we examined the cellular localization of an unrelated but highly conserved protein, designated as pErR, (Slomiany, Woldehawariat and Petryshyn, manuscript in preparation). Immunostaining with anti-pErR serum produced to highly purified protein indicated that

pErR was confined to a non-nuclear compartment in the absence of laminin (Fig. 2E) and this localization was unaltered by culturing on laminin-coated coverslips (Fig. 2F). Pre-immune serum, stained from the same animal prior to antigen introduction showed no staining, indicating that the anti-pErR serum was specific (data not shown). Hence, it is unlikely that in our model system laminin elicits a generalized non-physiologic effect on nuclear-localization of proteins.

Laminin-Dependent Translocation of Nucleolin

Since it has been shown that nucleolin can translocate (shuttle) between the cytoplasm and nucleus (10,12), we investigated whether the laminin-induced nuclear localization of nucleolin in IEC-6 cells was the result of increased translocation from a non-nuclear compartment. Within one hour following plating, when the cells still appear rounded, nucleolin was present in both the cytoplasm and nucleoli and the level of nucleolin each compartment was similar, in the absence or presence of laminin (Fig. 3A and B). After 5 hr, nuclear staining decreased to a similar extent both in the presence and absence of laminin (Fig. 3C and D), and the cells showed a modest increase in cytoplasmic staining (Fig. 3D). By 7 hr, nuclear staining of nucleolin was further diminished in the absence of laminin with an increase in cytoplasmic staining (Fig. 3E). On the other hand, an increase in nuclear nucleolin was observed in the presence of laminin (Fig. 3F). These results suggest that nucleolin initially translocates from the nucleus to the cytoplasm. However, following 7 hr of exposure, laminin promotes nuclear localization of nucleolin largely because of an increase in translocation from the cytoplasm to the nucleus. Following 15 hr in the absence of laminin, essentially all of the nucleolin was found localized to cytoplasmic compartments with little detectable nuclear nucleolin (see Fig. 1A). However, in the presence of laminin, the majority of the nucleolin was found tightly associated with nucleoli structures and it was diminished in non-nuclear compartments (see Fig. 1B).

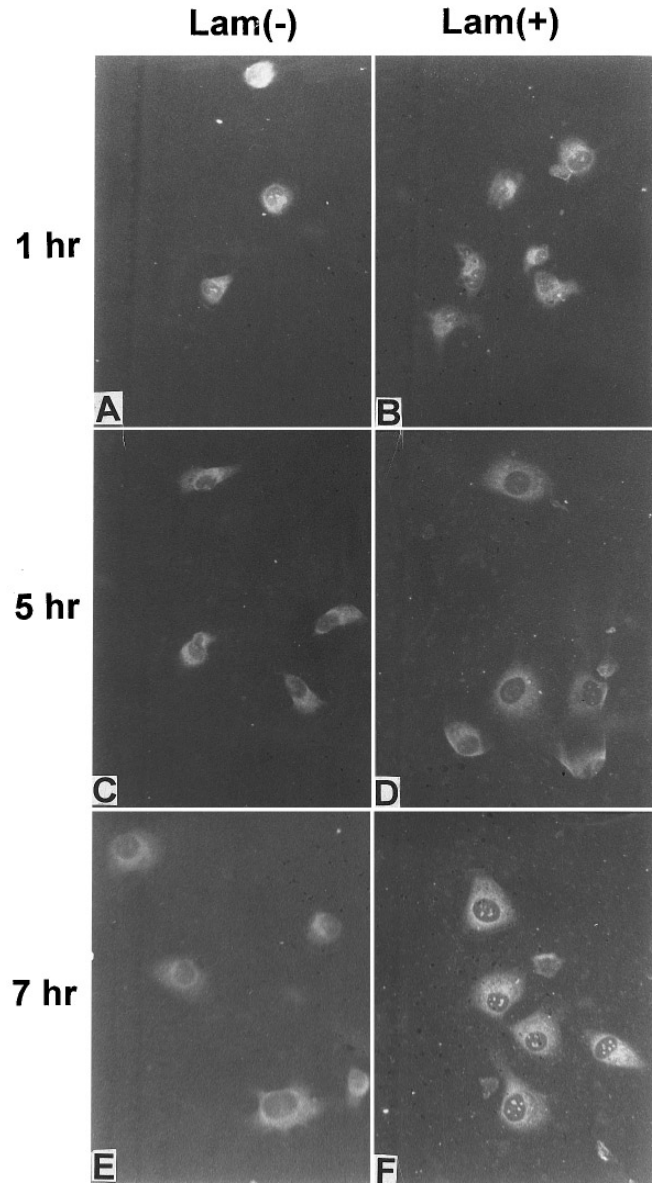

FIG. 3. Time course of laminin-dependent translocation of nucleolin. Cell culture and immunofluorescent staining were performed as described in figure 1. Panels A, 1 hr; C, 5 hr; E, 7 hr were immunostains of IEC-6 cells cultured on uncoated coverslips. Panel B, 1 hr; D, 5 hr; and F, 7 hr were immunostains of IEC-6 cells cultured on laminin-coated coverslips.

Cellular Redistribution of Nucleolin in Response to Laminin

The data in figure 4 indicates that a single band at 110 KDa previously identified as nucleolin (26) was immunologically detected in the cytoplasmic and nuclear fractions prepared from IEC-6 cells cultured in the presence or absence of laminin. The 110 KDa protein was not detected with pre-immune serum (data not shown). A redistribution of nucleolin was observed among the cytoplasmic and nuclear compartments. Within 6 hr of plating, nucleolin was detected in the nuclear and in the cytoplasmic fraction of the cells cultured on laminin coated plates. A further redistribution occurred with longer exposure to laminin such that by 15 hr, nucleolin was largely localized to the nucleus, while the cytoplasmic fraction became diminished in nucleolin content (Fig. 4). In contrast, little or no nucleolin could be detected in the nuclear fraction from cells cultured for 6 or 15 hr in the absence of laminin. These observations are in good agreement with the laminin-dependent pattern of nucleolin redistribution observed after immunofluorescence staining (Fig. 1 and Fig. 3). These findings indicate that laminin promotes redistribution of

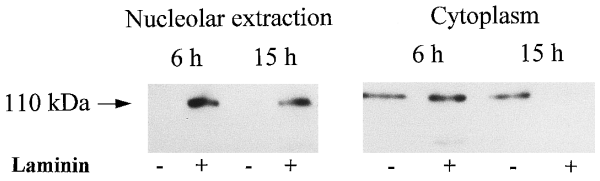

FIG. 4. Effect of laminin on the cellular distribution of nucleolin. IEC-6 cells were cultured on uncoated and laminin-coated plates. At the times indicated cells were lysed and subcellular fractions were prepared as described in Materials and Methods. Western blot analysis of using anti-nucleolin serum was performed as previously described (26).

nucleolin and suggests that this occurs because of a cytoplasm to nucleus translocation. However, it is possible that altered synthesis or stability of nucleolin in response to laminin may contribute to its apparent redistribution. This possibility could not be conclusively ruled out because inhibitors of protein and mRNA synthesis also prevented the attachment of IEC-6 cells to plastic or laminin-coated plates. Thus the contribution of laminin to synthesis or degradation of nucleolin could not be determined in our model system.

Effects of Laminin on Growth of IEC-6 Cells

Because of nucleolin's role in ribosome biogenesis (7,8) and laminin's effect on modulating proliferation of some cells (17), we examined the rate of proliferation of IEC-6 cells in the presence and absence of laminin and with the other adhesion molecules. As shown in figure 5, little or no proliferation of IEC-6 cells was observed in the absence of ECM molecules, or with poly-D-lysine, in a serum-free medium (not shown). Growth was substantially stimulated by culture on laminin and on collagen-coated plates and to a much lesser extent on fibronectin-coated plates, over the 4 day period. In each case, the observed change in proliferation was accompanied by the expected change in DNA synthesis indicating the extent of cell division (Fig. 6). Since a marked nuclear translocation of nucleolin was observed with laminin (Fig. 1 and 4), and to a progressively lower extent with collagen (Fig. 2C), but not with fibronectin or poly-D-lysine, these findings suggest that nuclear translocation of nucleolin, may be functionally linked to growth stimulation of IEC-6 cells by ECM components of which laminin may be the most effective.

DISCUSSION

The interaction of intestinal epithelium and mesenchyme is essential to induce enterocytic adhesion, differentiation and migration of both the developing gut and adult crypt-villus unit (19,20,27). Since basement membrane constitutes the interface between epithelial and mesenchymal cells, ECM is considered to be important for the dynamic cellular changes associated with these structures. There is ample evidence to indicate an active role for ECM in the regulation of the enterocyte differentiation, and several laminin binding proteins have been identified, including integrin, APP and LBP110 (25,28,29). A direct interaction between nucleolin and one of the laminin epitopes, IKVAV has been recently demonstrated in vitro (24,25), suggesting a possible link between nucleolin and ECM in cell regulation.

Our studies show that nucleolin is present in the cytoplasmic and nuclear compartments of IEC-6 cells. This finding is consistent with other reports indicating that nucleolin is not confined to the nucleus but is also localized in the cytoplasm (10,12). What is striking is that cells cultured for 15 hr on laminin-coated plates have less nucleolin associated with the cytoplasmic compartment than cells cultured in the absence of laminin. Moreover, most of the nucleolin was localized to the nucleoli structure of the nucleus. This result suggest that laminin enhances the translocation of cytoplasmic nucleolin to the nucleus.

The presence of nucleolin on the cell surface has been recently inferred (13). Direct contact between laminin

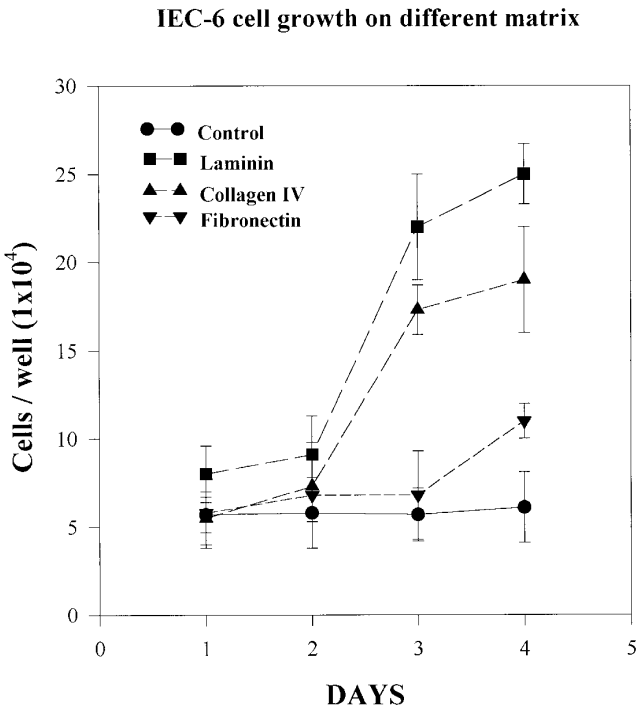

FIG. 5. Effect of ECM on the proliferation of IEC-6 cells. Culture dishes (24-well) received no treatment or were coated with laminin (70 μg/ml), collagen IV (70 μg/ml), or fibronectin (70 μg/ml) as described in Materials and Methods. Cells (5×10^4/well) were seeded in serum-free medium containing DMEM and 0.1 U/ml insulin. Cell number was determined by counting as described in Materials and Methods at the days indicated. Each data point represents the average (±S.E.) of triplicate determinations using separate wells.

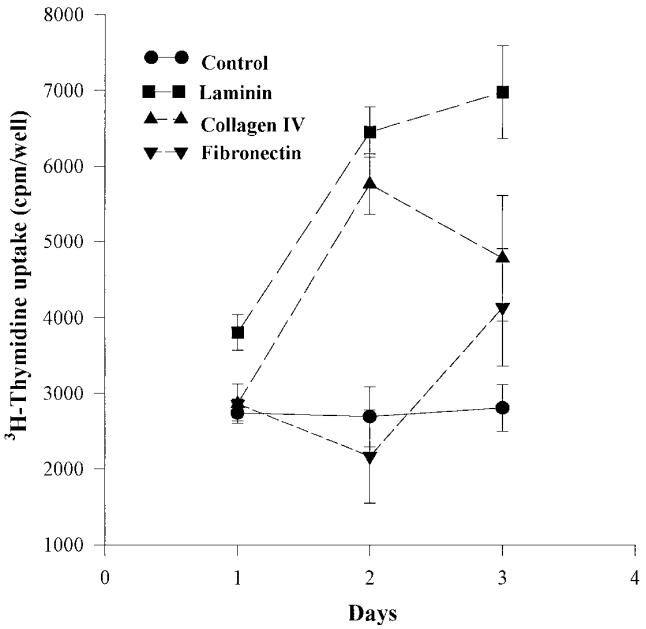

FIG. 6. Effect of ECM on DNA synthesis in IEC-6 cells. Cells (5×10^4) were seeded on coated and uncoated 24-well plates as described in Figure 5 and incubated in serum-free medium containing DMEM and 0.1 U/ml insulin for 24 hr. At the days indicated, plates were washed in PBS and the media was replaced with fresh DMEM containing ^{3}H-thymidine (1 μ Ci/ml, SA = 50 Ci/mmol, Sigma). Following a 4 hr pulse, plates were washed three times with 10% trichloroacetic acid and the samples were further treated with 0.2 NaOH (200 μl). Acid insoluble radioactivity was determined by scintillation counting. Values shown are the average (±S.E.) of triplicate determinations using separate wells.

and nucleolin would be the simplest explanation for the mechanism responsible for the redistribution of nucleolin. However, in our studies, we observed little or no nucleolin immunostaining on the surface of non-permeable IEC-6 cells (data not shown). Attempts at detecting surface membrane associated nucleolin of labeling with biotin were also unsuccessful. A similar observation has been made in HeLa cells using a different anti-nucleolin antibody in which ecto-kinase can phosphorylate the surface associated nucleolin but no immunostaining of this protein could be detected on the outer surface of the cell membrane (13). The could be explained if only a few molecules of nucleolin are localized on the outer cell surface and are thus difficult to detect or that the antibody may not recognize the protein because the antigenic sites of nucleolin may be masked when it is localized to the cell membrane. This may depend on the lipid composition of the cell membrane. It has been shown that a monoclonal anti-nucleolin antibody could detect a truncated nucleolin expressed on rat kidney tumor cell line but fail to identify the same truncated nucleolin expressed in human kidney tumor cell line, implying masking of epitopes. It is also possible that nucleolin may be localized to the cytoplasmic surface of the plasma membrane in these cells and are not readily detected by antibody or cross-linking with biotin in non-permeabilized cells. Finally, laminin may promote nuclear translocation of cytoplasmic nucleolin by an indirect mechanism.

We also observed that the stimulatory effect of laminin on the proliferation of IEC-6 cells in serum-free medium was greater than of collagen IV, fibronectin and poly-D-lysine. This is consistent with other studies demonstrating stimulation of proliferation of gastrointestinal epithelial cells by laminin during epithelial cell reconstitution and wound healing (19,20,30,31).

The translocation of nucleolin may be a necessary step for cell proliferation. One of the earliest events to occur following stimulation of cell proliferation is a change in the rate of ribosome precursor synthesis. Nucleolin has an essential role in ribosomal biosynthesis and as a major constituent of nucleolar organizer regions (NORs) (9). It has been well established that an active NORs is necessary for the transcription of the ribosomal genes, and for cell proliferation. In this study, we show that laminin has two effects on IEC-6 cells. It stimulates cell proliferation following 48 hr of culture and it specifically signals the nuclear localization of nucleolin within 15 hr of exposure to laminin. These findings raise the possibility that the two events are related and that laminin functions to coordinate these two events.

Further studies are needed to elucidate both the mechanism by which laminin interacts with nucleolin, and the mechanics of the translocation process in IEC-6 cells and in other anchorage-dependent cells. The findings of an ECM component causing direct translocation of a nuclear protein to its site of action in the nucleus may represent a mechanism by which laminin regulates the growth of IEC-6 cells.

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