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Collagen Receptors Mediate Early Events in the Attachment of Epithelial (MDCK) Cells

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Summary. Madin-Darby canine kidney (MDCK) cells kept in suspension culture for 12–15 hr displayed high-affinity binding sites for ^{125}I-lathyritic (soluble) collagen (120,000/cell, K_D = 30 nM) and preferred collagens types I and IV over laminin or fibronectin as substrates during the first hour of attachment. On the other hand, after 4 hr, attachment to all four substrates was equally efficient. Upon challenge with a collagen substrate, the high-affinity sites were rapidly recruited on it ($T\frac{1}{2}$ = 6 min). Their occupancy by soluble collagen triggered the exocytosis of a second large population of low-affinity collagen binding sites that included laminin and seems to be involved in a second cell-attachment mechanism. These results are compatible with a two-step model of MDCK cell attachment to the substrate: first, via high-affinity collagen binding sites, and second, via laminin of cellular origin.

Key Words epithelia · MDCK cells · cell-substrate interaction · collagen receptor · epithelial polarity · laminin

Introduction

The role of the substrate in the organization and differentiation of epithelial tissues has been increasingly recognized (*see*, e.g.: Kleinman, Klebe & Martin, 1981; Wicha et al., 1982; Gatmaitan et al., 1983; Enat et al., 1984; Lee et al., 1985; Parry et al., 1985). However, it is still unclear how cellular and extracellular factors cooperate to generate and maintain the asymmetry (polarity) of plasma membrane components, or the specific epithelial phenotype. While tight junctions and vectorial delivery mechanisms play a role in the maintenance of polarity in confluent monolayers (U & Evans-Layng, 1982; Herzlinger & Ojakian, 1984; Matlin & Simons, 1984; Misek, Bard & Rodriguez-Boulan, 1984; Pfeiffer, Fuller & Simons, 1985; Rindler et al., 1985), cell-substrate interactions appear to be essential in the initial polarization of previously unpolarized cells. Thus, epithelial cells in suspension culture lose the asymmetric distribution of their surface components (Pisam & Ripoche, 1976; Ziomek Schulman & Edidin, 1980), but regain it upon attachment to the substrate (Rodriguez-Boulan, Paskiet & Sabatini, 1983; Balcarova-Stander et al., 1984; Ingber, Madri & Jamieson, 1986; Vega-Salas et al., 1987*a*). In some cases, the polarity of already established confluent epithelial monolayers can be reversed by adding substrate material, such as collagen gels, to the apical surface (Chambard, Gabrion & Mauchamp, 1981; Hall et al., 1982; Chambard et al., 1984). The organizational role of the substrate is also evident in other important functions such as regulation of membrane protein synthesis and distribution (Kabat et al., 1985; Vega-Salas, Salas & Rodriguez-Boulan, 1987*b*). Even epithelial cell proliferation is "anchorage dependent" in most epithelial cell lines (for a review, *see* Yang & Nandi, 1983).

Cell lines in tissue culture that reproduce polarity properties of parental epithelia, such as Madin Darby canine kidney (MDCK) (Leighton et al., 1969; Misfeldt, Hamamoto & Pitelka, 1976; Cereijido et al., 1978; Handler, Perkins & Johnson, 1980), provide excellent systems to study the origin of epithelial cell polarity and differentiation (Rodriguez-Boulan, 1983*a*; Simons & Fuller, 1985), and, in particular, the role of early cell-substrate interactions. Little and contradictory information is available about the molecules that mediate early cell-substrate interactions in epithelial cells in vitro. While Terranova, Rohrbach & Martin (1980) reported that PAM-212 (epithelial) cells require laminin (endogenous or exogenous) for attachment after 4 hr, Rubin and coworkers (1981) reported that hepatocytes adhere directly to collagen in periods as short as 30–60 min, and Johansson and coworkers (1981) have reported that the same cells attach to laminin and fibronectin within the initial 80 min. Ideally, a cell line expressing all the attachment mechanisms that may be working together in vivo would give a more complete insight about the rela-

Table 1. Early attachment of MDCK cells to various substrates

Protein added to each support	Number of cells attached to:		
	Agarose (1 hr)	Plastic	
		(1 hr)	(4 hr)
Type I collagen			
Total	30 ± 9	23 ± 6	23 ± 5
Spreading	15 ± 9	12 ± 3	19 ± 5
Type IV collagen			
Total	24 ± 7	20 ± 5	24 ± 9
Spreading	8 ± 5	3 ± 1*	21 ± 12
Laminin			
Total	6 ± 8	9 ± 6*	31 ± 5*
Spreading	0.4 ± 0.5*	3 ± 2*	23 ± 11
Fibronectin			
Total	3 ± 3*	10 ± 3*	26 ± 10
Spreading	0*	3 ± 2*	23 ± 9
None			
Total	0.2 ± 0.4*	0.8 ± 0.9*	4 ± 3*
Spreading	0*	0*	0
Albumin			
Total	ND	1.2 ± 4*	13 ± 4*
Spreading		2 ± 1*	4 ± 2*

Different proteins (up to 1 mg/ml) were mixed with 3% ultralow gelling point agarose. The same proteins were dissolved in distilled water from stock solutions up to a final concentration of 1 mg/ml and applied to 35-mm tissue culture dishes ("Plastic"). MDCK cells were kept overnight in suspension culture, gently spun onto these substrates, and incubated at 37°C. After 1 or 4 hr the loose cells were removed and the attached cells counted.
* Indicates that the mean is significantly different ($P < 0.05$) from the corresponding mean with type I collagen.

tive role of each substrate receptor in the complex process of epithelial tissue organization. In this study we describe the initial attachment of MDCK cells as a multi-step process involving collagen, laminin and fibronectin receptors. A population of collagen binding sites in MDCK cells mediate early cell-substrate interactions and induce the exocytosis of endogenous cellular laminin.

Materials and Methods

Materials

Materials (sources) were as follows: EDTA (Fisher, Fair Lawn, N.J.); trypsin (Millipore Corp., Freehold, N.J.); egg white trypsin inhibitor, hyaluronidase, neuraminidase, chondroitinase ABC, heparinase, EGTA (Sigma, St. Louis, Mo.), heparitinase (Seikagaku Kogyo, Tokyo, Japan); bovine plasma fibronectin (Bethesda Research Laboratories [BRL], Gaithersburg, Md.); laminin (BRL); rabbit antisera to mouse laminin (BRL). Rabbit antisera to fibronectin was a gift from Dr. Celso Bianco, N.Y. Blood Bank, N.Y.C. Purity of extracellular matrix (ECM) components was determined by three criteria: (a) biochemical, by electrophoresis in polyacrylamide gels (SDS-PAGE) and Coomasie blue staining, (b) immunological, by reactivity with specific antibodies on immunoblots (*see below*), (*data not shown*, copies of gels and immunoblots available upon request), and (c) functional by induction of cell attachment to tissue culture plastic (Table 1). Interspecies cross reactivity of both antibodies was tested on dog kidney semi-thin frozen sections by immunofluorescence.

Collagen Iodination

Lyophillized lathyritic rat skin collagen (LRSC) (a gift from Dr. A.H. Reddi, National Institutes of Health, Bethesda, Md.) (Barrow, Simpson & Miller, 1974; Reddi & Sullivan, 1979) was diluted in 0.01 M acetic acid to obtain a stock solution (usually 1 mg/ml, 2 mg/ml when higher final concentrations were desired). Maximal solubility of LRSC at neutral pH from lyophillized material was ~1 mg/ml. LRSC (50 μg) was coupled to 2.5 mCi of ^{125}I (New England Nuclear, Boston Mass.) with the chloramine *T* procedure (Greenwood, Hunter & Glover, 1963). The iodinated protein [Rat type I collagen contains 3.2 tryosine residues per 1000 amino acid residues (Lowther, 1963)] was separated from the free iodide by filtration through a 50-cm long Sephadex G-100 (Pharmacia, Piscataway, N.J.) column. The first peak eluting from the column was analyzed by SDS-PAGE. The gels were fixed in 45% methanol/1% acetic acid, stained with 0.1% Coomasie blue, dried and exposed to *X*-ray film (Kodak XAR-5) for autoradiography. Only preparations showing the typical alpha, beta and gamma collagen pattern and no detectable radioactivity in the front were used. Stock and iodinated LRSC were stored at −20°C.

Cell Culture

MDCK cells were grown in Dulbecco's MEM, 10% FCS, as described elsewhere (Rodriguez-Boulan, 1983*b*). For suspension culture, MDCK monolayers were dissociated with 0.1% trypsin, 0.5 mM EDTA, spun, resuspended in Dulbecco's MEM (D-MEM) (Gibco, Grand Island, N.Y.) containing 10% fetal calf serum (FCS), spun and incubated overnight in serum free D-MEM 0.2% BSA (5×10^5 cells/ml) in a siliconized spinner flask. These conditions allowed suspension cultures with low proportion of dead cells (usually less than 10%, as determined by trypan blue exclusion test), few aggregates, and maximum binding of LRSC (reached 10–15 hr after dissociation). Immediately before the experiments, the suspension cultures were filtered through a 15-μm nylon mesh (Tetko, Elmsford, N.Y.). Confluent MDCK monolayers were grown on nylon net discs (2 μm pore, Tetko) mounted on plastic rings, and transepithelial resistance (TER) was measured as described in a previous work for gallbladder epithelium in vitro (Salas & Moreno, 1982). Only those filters showing TER levels >50 $\Omega \cdot cm^2$ were used to study the binding of LRSC added from the apical or from the basal side. The leakage of IgG in MDCK cell monolayers showing transepithelial resistance of this magnitude was shown to be very small (Salas et al., 1986). Monolayers of BHK (fibroblastic) cells prepared in the same way were used as a control of these experiments.

Attachment Assay

A fixed number of cells (typically 2×10^5 cells/Petri dish) in suspension culture (in Dulbecco's MEM, 0.2% BSA, 30 mM

HEPES, pH 7.4) were pipetted into a Petri dish either pretreated with protein stock solutions or containing agarose/protein gels. The cells were immediately spun in a Sorvall T600 centrifuge at 20–30 × *g* for 40 sec (plus the acceleration/deceleration times that contributed some additional 40 sec) in order to synchronize the attachment. The Petri dishes were usually incubated for 60 min at 37°C in a water jacketed incubator under a 5% CO_2/air atmosphere. The incubation times or temperatures were changed in some experiments. After incubation, each Petri dish was washed three times with 1 ml PBS, gently shaking three times with each wash. The washing solution was aspirated and the attached cells were immediately counted under an inverted microscope with phase optics. The washes mostly removed cell aggregates formed during or after the centrifugation. These aggregates never attach to the substrate because they usually polarize with the apical domain out (*see* Rodriguez-Boulan et al., 1983). Preliminary controls showed that the nonaggregated cells that remain attached are viable and capable of forming a confluent monolayer indistinguishable from the parental MDCK monolayer. For counting, the following conventions were used: (i) cells shifting in the microscope field were considered as nonattached, and were not counted; (ii) cell clusters (usually 2–4 cells) were counted as single cells. Four to eight fields were selected at random per Petri dish, counted and averaged. The results, in cells per field, were normalized as per cent of cells plated. In preliminary experiments we found that the percent of attached cells was a linear function of the number of plated cells up to 10^5 cells/cm^2 (about the cell density at confluency). In most experiments the cells were plated at a density of 1.2 to 3.7 × 10^4 cells/cm^2.

An important assumption when using agarose/protein gels regards the final concentration of the protein. The diffusion of soluble LRSC out of a gel was determined in independent experiments by mixing [^{125}I]LRSC with agarose and measuring the time course of diffusion in a system similar to those used for the attachment assay. It was found that only 16% of the total initial radioactivity of the gel was in the solution after 5 hr. Therefore, in order to validate this assumption, experiments on agarose/protein gels were conducted in periods no longer than 1 hr. The second assumption is that the volume occupied by the agarose itself is negligible as compared with the total volume, in other words, that the trapped solution volume is approximately equal to the total gel volume. Since the agarose does not significantly change volume when gelling and dry material represents 3% of volume, the assumption is valid. In fact, the structure of agarose gels has been described as loose networks of galactose-derived polysaccharides, with interstitial solution trapped in spaces of 50 Å average equivalent diameter (Rees, 1969).

Binding Assay

Cell suspensions were supplemented with [^{125}I]LRSC to a final activity of 2 × 10^6 to 6 × 10^6 cpm/ml and incubated for 5–40 min (kinetic experiments) or for 50 min (equilibrium experiments). In order to separate bound from free ligand, cells were centrifuged (1400 × *g*, 1 min) through a 2-ml cushion of 10% (wt/vol) dextran T70 (Pharmacia) (specific gravity D_{20}^{20} = 1.039) in PBS (140 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.5 mM $MgCl_2$ and 0.05 mM $CaCl_2$). This procedure resulted in a pellet composed only of dye-excluding cells. Dead cells remained at the medium-dextran interface. The supernatant medium was immediately removed and the dextran cushion was aspirated in three steps, at ~30 sec intervals, to avoid the contamination of the pellet with free ligand draining off the walls of the tube. The bottom of the tubes, containing the pellets plus 200–400 μl dextran solution were cut with a razor blade and their radioactivity measured in a Packard gamma spectrometer. Whenever necessary, the activities of the samples were normalized for isotope decay. To determine the specific activity of the ligand in the suspension cultures, aliquots were counted directly. For dose-response studies, excess cold LRSC was mixed in various proportions with a fixed amount of iodinated ligand containing only trace amounts of carrier LRSC. Since the cells had to be kept very diluted in suspension culture to avoid aggregation, they bound only small proportions of the labeled ligand. The composition of the bound ligand was analyzed by SDS-PAGE and autoradiogram. It was found that the bound ligand was undergraded collagen.

Quantitation of Surface and Intracellular Laminin by Radioimmunoassay

12–15 hr suspension cultures of MDCK cells were exposed to various concentrations of LRSC for 40 min, fixed with 2% formaldehyde (freshly prepared from paraformaldehyde) in PBS, quenched with 50 mM NH_4Cl in PBS and processed for indirect RIA. Intracellular laminin was quantitated in fixed samples permeabilized with 0.2% Triton X-100 in PBS. All antibody incubations were carried out in 1% BSA-PBS: biotin coupled anti-laminin antibody (biotin coupled BSA in control samples) in the first step and [^{125}I]avidin (400,000 cpm/ml, New England Nuclear, Boston Mass.) in the second one. After three washes the pellets were counted in a Packard gamma spectrometer.

Immunofluorescence

Procedures for semi-thin frozen sections and immunofluorescence have been described elsewhere (Salas et al., 1986; Vega-Salas et al., 1987*a*).

Immunoblot and Immunoprecipitation

For immunoprecipitation (IP) experiments, MDCK cells were metabolically labeled overnight in suspension culture in MEM containing 10% of the normal medium concentration of methionine and cysteine (1 mM), 33 μCi/ml of [^{35}S]methionine and 33 μCi/ml of [^{35}S]cysteine.

Confluent monolayers or pellets of MDCK cells in suspension culture were scraped and homogenized by 50 passages through the blue tip of a 1-ml Eppendorff pipette in the following extraction buffer: 50 mM HEPES, 1 mM EGTA, 1 mM PMSF, 1 mM aprotinin, 10 μg/ml pepstatin, 15.7 μg/ml benzamidine, 0.5% Nonidet P40, 0.1% sodium deoxycholate, 0.1% ovoalbumin, pH 7.5. The nuclei and cell debris were spun down in an Eppendorff centrifuge (12,000 × *g* for 2 min) and the supernatants were stored at −70°C.

For immunoblots, the extract from ~3 × 10^7 cells was supplemented with 0.5% SDS and sample buffer, and run in a 10-cm wide lane in 3–10% polyacrylamide gradient gels using the system of buffers described by Laemmli (1970). The gels were electroeluted on nitrocellulose filters according to the technique described by Towbin, Staehelin and Gordon (1979). The nitrocellulose filters were then saturated with 1% BSA in PBS, cut into 1-cm wide strips, and each strip was incubated with a polyclonal rabbit anti-laminin antibody, followed by [^{125}I]protein

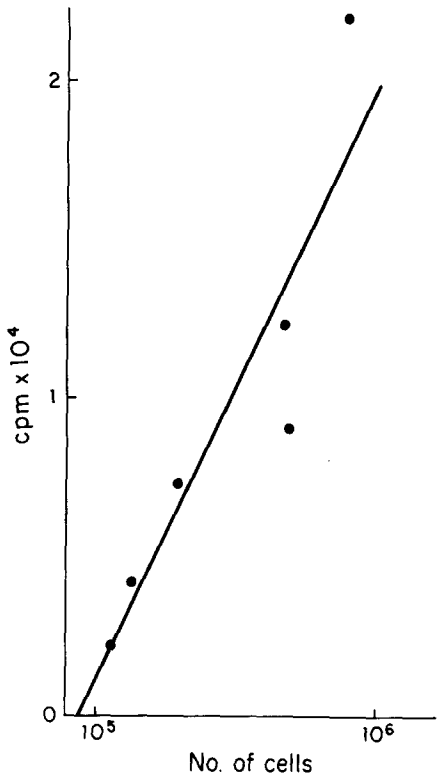

Fig. 1. [^{125}I]LRSC binding to MDCK cells in suspension culture. MDCK cells were kept in suspension culture for 12–15 hr, incubated for 40 min in the presence of [^{125}I]LRSC (the carrier collagen concentration was approximately 0.1 nM and the specific activity 1.1×10^{10} cpm/nmol) and spun through a 10% dextran T70 cushion. Preliminary determinations showed that viable (trypan blue excluding) cells in suspension have a specific gravity of ~1.07 g/ml, equivalent to the specific gravity of a 15% dextran solution, while dead cells have a specific gravity of 1.025 g/ml (equivalent to 3% dextran). The cpm per cell pellet are plotted against the number of viable cells per sample, as determined by trypan blue exclusion. Each point represents a duplicate independent observation. Linear regression coefficient: 0.82, intercept: -9.8×10^2 cpm

A (ICN Radiochemicals, Irvine Calif.), washed, dried and exposed to *x*-ray film.

For IP, the extract from ~3×10^6 cells was supplemented with a 10× stock solution up to the following final concentrations (IP buffer): 0.01 M sodium phosphate, 0.1 M NaCl, 0.1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 0.2% ovoalbumin. Then each sample received sequentially: 20 μg/ml anti-laminin antibody and ~100 μg/ml protein A coupled to Sepharose 4B (Sigma), and incubated at room temperature for 1 hr in each step. The Sepharose beads were then washed three times in IP buffer and three more times in IP buffer supplemented with 1.5 M KCl. After a final wash in IP buffer, the antibody associated to the beads was dissociated by sonication in 10 mM Tris Cl, 1% SDS, 1% 2-mercaptoethanol, pH 7.5. This supernatant was run in SDS-polyacrylamide gels as described above. The gels were fixed in 40% methanol, 10% acetic acid; equilibrated in Enhance (T.M., New England Nuclear, Boston Mass.), dried and exposed to x-ray film at −70°C.

Results

Attachment of MDCK Cells During the First Hour Is More Efficient on Collagen than on Laminin or Fibronectin

In order to identify patterns of preference in the early interactions of MDCK cells with the substrate, we studied the efficiency of attachment and spreading on various extracellular matrix (ECM) substrate materials: type I collagen, type IV collagen, laminin and fibronectin. These molecules were used either bound to tissue culture plastic, as described by Rubin et al. (1978), or mixed with ultra-low gelling point agarose (*see* Materials and Methods). The latter assay allows an accurate estimate of the substrate "concentration" during short (1 hr) incubation periods; at longer times the amount of substrate molecules that diffuse into the medium becomes significant (16% at 5 hr). During the first hour of attachment, MDCK cells showed equal clear preference for type I and type IV collagens over laminin or fibronectin (Table 1). Spreading, however, was somewhat slower on type IV collagen (50 and 30% of the attached cells, respectively). Attachment and spreading on laminin and fibronectin, although very low during the first hour, increased significantly after a 4-hr incubation. Spreading cells on laminin resulted in a strikingly different morphology than on the other substrates: the cells were flat but lacking "dendritic" prolongations, typically observed in cells growing on fibronectin or collagens. Appropriate controls showed that the differences described above are, indeed, substrate related, since all the molecules used were pure (as seen by SDS-PAGE), were recognized by specific antibodies obtained from different sources, and all of them had strong biological activity, i.e., they induced cell attachment levels 10 to 100 times above background (Table 1, negative controls with albumin or no protein). These results suggested that early attachment of MDCK cells is mostly mediated by collagen receptors and that laminin and/or fibronectin receptors are involved in a secondary phase.

Equilibrium Binding of ^{125}I-Soluble Collagen Molecules to MDCK Cells in Suspension Culture

The presence of collagen receptors in MDCK cells in suspension culture was demonstrated in binding assays with [^{125}I]lathyritic rat skin collagen (LRSC, salt extractable type I collagen obtained from lathyritic rats (*see* Barrow et al., 1974)) as ligand. The

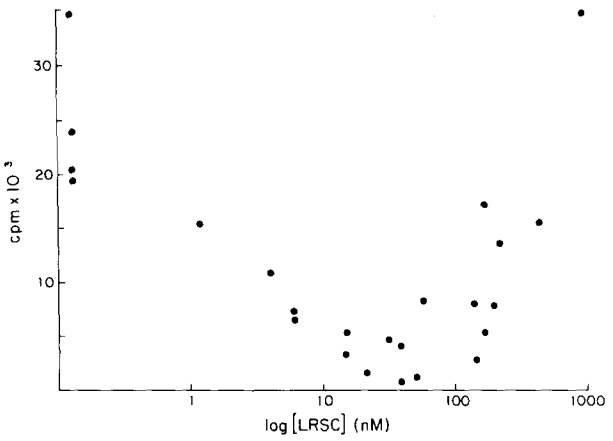

Fig. 2. Equilibrium binding of [^{125}I]LRSC to MDCK cells in suspension culture. MDCK cells were kept in suspension culture for 12–15 hr. Parallel samples (4 ml each) from the same suspension were incubated with a fixed amount of [^{125}I]LRSC (typically 2×10^6 to 6×10^6 cpm/ml) and variable amounts of cold LRSC for 40 min, spun through the dextran cushion, and counted. The raw data in cpm/10^6 cells from seven independent experiments are plotted against the logarithm of LRSC concentration. The only correction included was the normalization for the number of cells, which was validated by the linear relationship between binding and cell number shown in Fig. 1. Note that: (i) cold LRSC displaces at least 80% of the binding between 0 and 50 nM, indicating a reasonably low level of nonspecific binding; and (ii) there is an ascendent phase of the plot in the higher concentration range (50 to 800 nM)

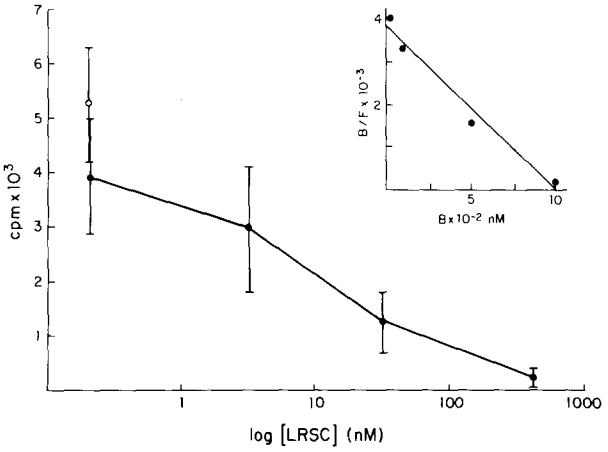

Fig. 3. Equilibrium binding of [^{125}I]LRSC or MDCK cells fixed with formaldehyde. MDCK cells were kept in suspension culture as described above and either fixed (●) or not (○) with an equal volume of 4% formaldehyde (freshly prepared from paraformaldehyde, PFA) in PBS (final concentration 2% PFA) for 30 min. The fixed cells were washed in PBS by centrifugation, quenched with 50 nM NH_4Cl, spun and resuspended in standard MEM. All samples were incubated with a fixed amount of [^{125}I]LRSC ($\sim 10^6$ cpm/ml) and variable amounts of cold LRSC; therefore, the specific activity varied from 5×10^8 to 10^6 cpm/nmol. The averages of four independent experiments, expressed in cpm/10^6 cells, are plotted against the logarithm of LRSC concentration. Note that: (i) cold LRSC displaces at least 92% of the binding, indicating a reasonably low level of nonspecific binding; and (ii) there is no ascendent phase of the plot in the higher concentration range (50 to 330 nM). Insert: Scatchard plot of the same data. Specific bound LRSC concentration was calculated by subtracting the nonsaturable component

binding of [^{125}I]LRSC was a linear function of the number of live cells in the sample (Fig. 1). Preheating of the ligand to 60°C resulted in a significant decrease of LRSC binding (*not shown*).

The equilibrium binding of LRSC, studied by increasing the cold LRSC concentration while keeping the [^{125}I]LRSC concentration constant, displayed a strikingly biphasic behavior (Fig. 2). Between 0 and 40 nM LRSC, the binding of [^{125}I]LRSC decreased. However, after reaching a minimum at ~40 nM, the amount of bound radioactive ligand increased with no evidence of saturability up to 800 nM (0.24 mg/ml), which is close to the maximum solubility of the LRSC at neutral pH (~1 mg/ml when lyophillized material is diluted directly in saline solution).

We speculated that the increase in binding at higher LRSC concentrations might be caused by: (i) artifactual LRSC polymerization on the cell surface; (ii) exocytosis of a new population of low-affinity binding sites triggered by the occupancy of the high-affinity binding site population; (iii) increase in receptor-mediated endocytosis at higher LRSC collagen concentrations.

The first possibility was eliminated by binding experiments to fixed MDCK cells (Fig. 3). Fixation decreased LRSC binding by only 30% and displacement of binding by cold ligand occurred in the same range of concentrations as in nonfixed cells; however, the rise in binding at 50–800 nM LRSC was not observed. Furthermore, incubation of LRSC in the absence of cells at the same concentrations, temperature, ionic concentration and time showed no difference in its sedimentation coefficient (*data not shown*). Thus, the second phase of the equilibrium LRSC binding is a process requiring cell metabolism (such as any of the last two possibilities mentioned above) and not simply an artifactual surface phenomenon. The Scatchard analysis could be performed only for the data from fixed cells (Fig. 3, insert), and showed 1.2×10^5 receptors per cell with an apparent K_D of 30 nM.

Kinetics of [^{125}I]LRSC Binding to MDCK Cells in Suspension Culture

MDCK cells in suspension culture were exposed to trace amounts of [^{125}I]LRSC containing no cold LRSC. The binding reached a plateau after 20 min;

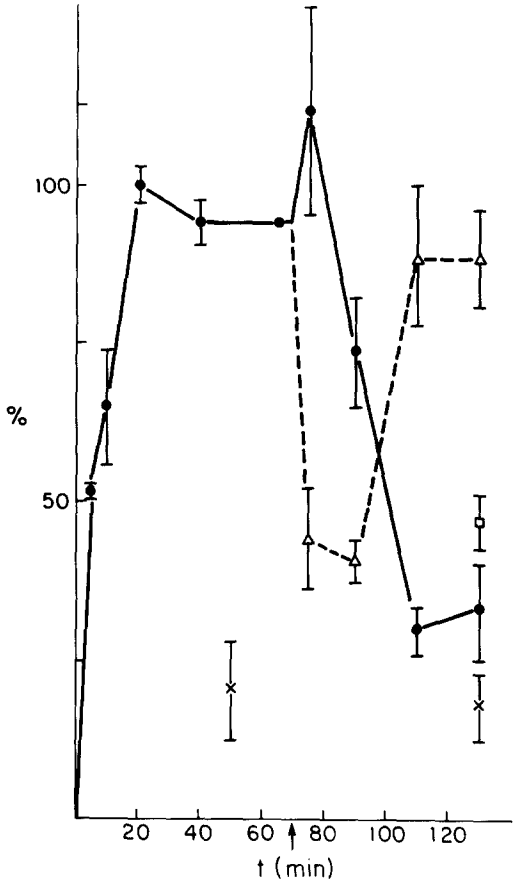

Fig. 4. Time course of [^{125}I]LRSC binding to MDCK cells in suspension culture. [^{125}I]LRSC (10^6 cpm/ml, ~0.1 nM) was added at time 0 to MDCK cells in suspension culture. At various times, 2 ml duplicate aliquots were taken and cells were immediately separated from the free ligand by centrifugation through a 10% dextran cushion. The mean values from three independent experiments are expressed as per cent of the 20 min value (●). At 40 min one set of duplicate aliquots was incubated in the presence of 1.32 U/ml collagenase for 10 min and then spun (×). After 60 min, the suspension was separated in two batches and cold LRSC was added (arrow) at two different concentrations: 30 nM (●) and 330 nM (△). At various times, aliquots were taken as described above. At 150 min, one set of aliquots from each concentration was incubated in the presence of collagenase as described before: 30 nM LRSC + collagenase (×) and 330 nM LRSC + collagenase (□). Note that a significant ($P < 0.05$) increase in the collagenase-insensitive component appeared in the higher LRSC concentration

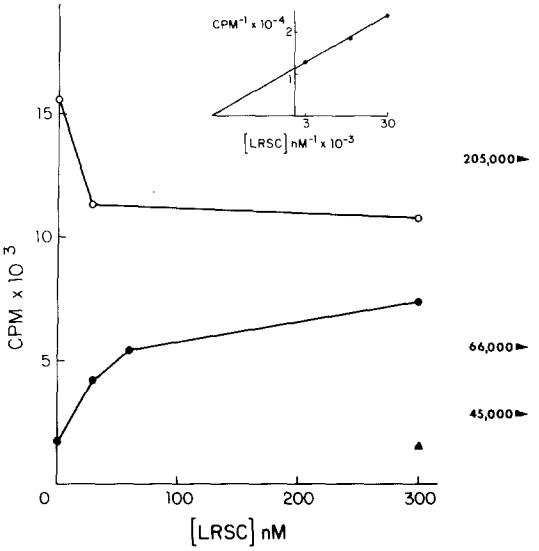

Fig. 5. Binding of soluble LRSC promotes the exocytosis of laminin by MDCK cells in suspension culture as determined by radioimmunoassay. MDCK cells were kept in suspension culture for 12–15 hr, incubated in the presence of soluble LRSC for 40 min at 37°C or at 4°C (▲), treated with 0.1 U/ml bacterial collagenase and fixed overnight with 2% PFA (final concentration). The cells were processed for radioimmunoassay in the presence (○) and in the absence (●, ▲) of 0.2% Triton X-100. The identification of laminin in MDCK cell extracts by the first antibody used in this experiment is shown by immunoblot [the lack of reactivity with the 440-kD subunit of laminin was presumable due to a poor transference because this subunit was found by immunoprecipitation; *see* Fig. 6]. The data from six independent experiments, in cpm, are plotted against the LRSC concentration. Note that the amount of laminin on the cell surface increases with the LRSC concentration while the whole cell content decreases, suggesting both externalization and secretion of laminin to the medium. When the cells were incubated at 4°C the externalization was abolished (▲). The same data, plotted in inverse coordinates (insert), was used to calculate an apparent K_D of 40 nM

at this time, 80% of the bound collagen was on the cell surface, as demonstrated by its accessibility to collagenase (Fig. 4). Addition of 30 nM cold LRSC at 60 min resulted in simple dissociation kinetics that soon reached an apparent plateau (Fig. 4, ●); most of the remaining bound collagen was also collagenase insensitive. On the other hand, the addition of 330 nM cold LRSC (Fig. 4, △) resulted in an early, significant dissociation of iodinated ligand, which was followed by a late increase in binding up to the level of the initial association plateau. 50% of this late association plateau was collagenase insensitive, indicating that only part of it was endocytic uptake. Using the association curve and the dissociation curve after addition of 30 nM LRSC (Fig. 4), K_D = 25 nM was obtained, similar to the K_D calculated from equilibrium binding data in fixed cells (30 nM).

The kinetic experiments on live cells, therefore, confirmed the equilibrium binding data from fixed cells. The late increase in [^{125}I]LRSC binding to MDCK cells at high LRSC concentrations corresponded to the secondary increase in equilibrium LRSC binding curve (Fig. 2). This increase can be explained by both ligand endocytosis, and the appearance of a new population of binding sites triggered by the occupancy of the high affinity sites. Since these sites could not be saturated with LRSC

concentrations up to 0.25 mg/ml (800 nM) they will be named hereafter as low affinity sites, although their affinity could not be determined. The nature of these new sites is explored in the next section.

LRSC Binding to MDCK Cells Induce the Exocytosis of Intracellular Laminin

Immunoblot experiments on MDCK cell extracts allowed the detection of laminin (Fig. 5). Fibronectin (*not shown*) was not detected by this procedure. Since laminin has one or more collagen binding sites (Woodley et al., 1983; Charonis et al., 1985), we investigated the possibility that it was exocytosed by MDCK cells in response to LRSC binding. MDCK cells in suspension culture were assayed for the presence of surface and intracellular laminin by indirect RIA using a monospecific polyclonal anti-laminin antibody. Total cellular laminin, quantitated in fixed cells treated with detergent was found to be 8.6 times higher than surface laminin in suspended MDCK cells (Fig. 5). Incubation of MDCK cells in suspension culture with increasing concentrations of LRSC resulted in a fourfold increase in surface laminin and a 1.6-fold decrease in the total cell laminin content (Fig. 5). By adding these two values, the amount of laminin exocytosed by MDCK cells in response to LRSC binding was calculated to be six times the original extracellular levels. Plotting these data on inverse coordinates (Fig. 5, insert) showed an apparent $K_D \sim 40$ nM, similar to the K_D's of the high affinity collagen binding sites derived from the kinetic and equilibrium binding experiments described above.

The exocytotic response to LRSC binding was also observed by immunoprecipitation of metabolically labeled laminin (Fig. 6). MDCK cells in suspension culture, prelabeled with [^{35}S] methionine and cysteine, were incubated in the presence of 300 nM LRSC and then exposed to proteinase K. Most of the laminin became accessible to protease (i.e., extracellular) after addition of collagen.

Frozen sections of MDCK cells grown on native rat tail collagen gels showed a thin layer of laminin between the cells and the substrate by immunofluorescence (*not shown*), indicating that laminin may mediate cell attachment when the monolayer is established.

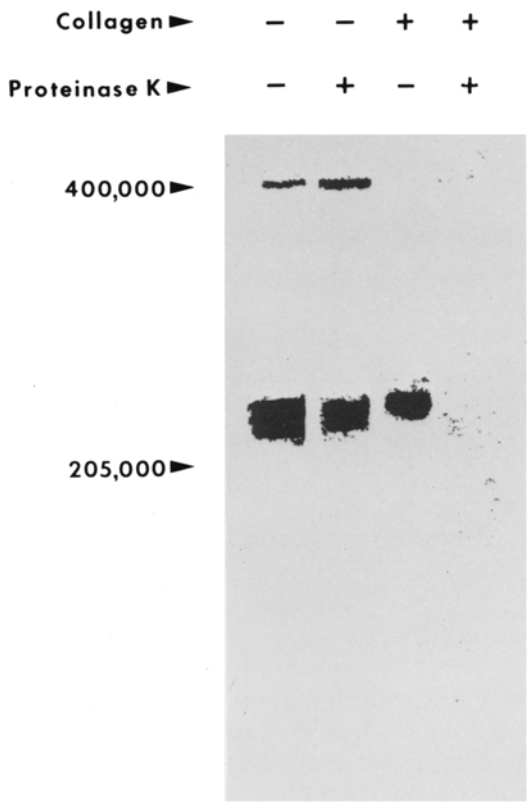

Fig. 6. Binding of soluble LRSC promotes the exocytosis of laminin by MDCK cells in suspension culture as determined by accessibility to protease. MDCK cells were kept overnight in suspension culture in MEM containing 10% of the normal medium concentration of methionine and cysteine (1 mM), 33 μCi/ml of [^{35}S]methionine and 33 μCi/ml of [^{35}S]cysteine. One half of the cell culture was incubated for 45 min in the presence of 300 nM collagen. Then, one half of each suspension was incubated in the presence of 0.16 U/ml proteinase K for 10 additional minutes at 4°C. The cells were cooled down on ice, spun and washed three times with cold PBS. The pellet was solubilized with extraction buffer, and the postnuclear supernatant from 3.5×10^7 cells per sample was processed for immunoprecipitation. The material obtained (usually 0.1% of the initial cpm) was electrophoresed in 3 to 10% gradient SDS-polyacrylamide gels using the buffer system described by Laemmli (1970). The gels were dried and exposed 1 week for autoradiography. Addition of soluble collagen caused the disappearance of the 440-kD laminin subunit and a noticeable decrease in the amount of the 220-kD laminin subunit (third lane). Note that proteinase K did not alter the content of laminin in unstimulated cells (second lane) but caused a significant decrease of cell-associated laminin in cells stimulated with soluble collagen (fourth lane). If the gel was over-exposed a faint band appeared at the 220-kD mol wt (*not shown*)

Involvement of Collagen Binding Sites in the Initial Attachment of MDCK Cells to the Substrate

The experiments shown in Table 1 suggested that early attachment of MDCK cells to the substrate was mediated by collagen sites. To study whether these sites are the same as those defined by the [^{125}I] LRSC-binding experiments, we studied the attachment of MDCK cells to agarose gels containing various concentrations of LRSC (Fig. 7). Since MDCK cells do not attach to agarose (Table 1), all attachment observed could be attributed to the LRSC.

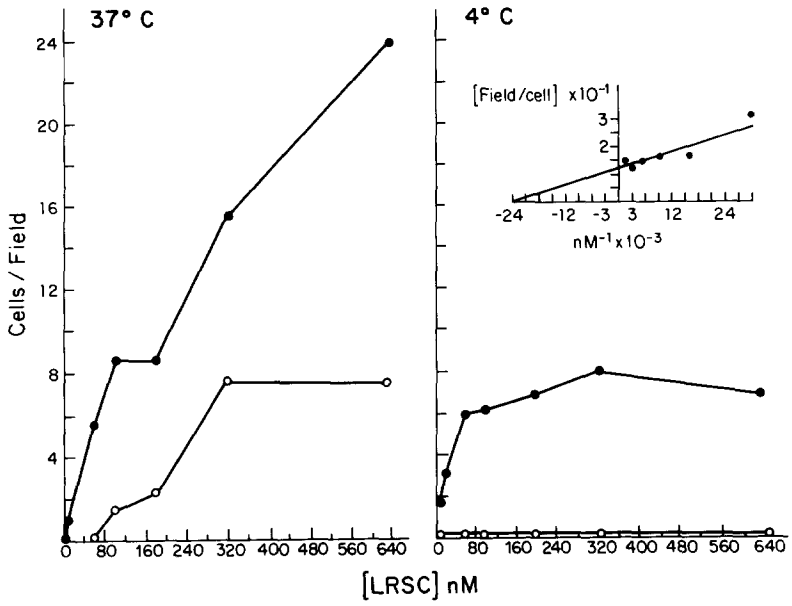

Fig. 7. Attachment of MDCK cells in suspension culture to 3% agarose containing various concentrations of LRSC. Various concentrations of LRSC were obtained diluting a stock solution in 3% low gelling point agarose at 37°C. MDCK cells (kept in suspension culture for 12 to 15 hr) were spun onto them (40 sec, 20–30 g) and incubated for 60 additional minutes at 37°C or at 4°C. The loose cells were washed out with PBS and the attached cells counted. The data are expressed as cells/field (typically 100–200 cells were plated) that attached (●) or spread (○) on the substrate as a function of LRSC concentration. Note that the cells did not spread at 4°C and that, at the lower temperature, cell attachment followed simple saturation kinetics. The same data plotted in inverse coordinates (insert) showed an apparent K_D of 41 nM

Increasing LRSC concentrations in the range 1–700 nM caused a continuous increase in the number of attached cells; detectable spreading onto the substrate started at 60 nM and increased up to a maximum of 30 to 50% of the total number of attached cells at the higher LRSC concentration (Fig. 7). In agreement with previous studies (Grinnell, 1978), attachment proceeded but spreading was blocked at low temperatures (Fig. 7). Attachment at 4°C was a saturable function of the LRSC concentration in the substrate, with an apparent K_D of 41 nM (Fig. 7, insert). A study of the temperature dependence of MDCK cell attachment to the agarose-collagen substrate (Fig. 8), demonstrated that a single mechanism was responsible for early cell attachment at both 39 and 4°C. The Arrhenius plot yielded an activation free energy of 8.3 kcal/mol, within the order of magnitude observed for lateral protein diffusion in biological membranes (Schlessinger et al., 1976). This result indicates that initial attachment of MDCK cells does not require cell metabolism and may involve the recruitment of the collagen-binding sites on the substrate.

The striking similarity of the K_D's for initial cell attachment, laminin exocytosis and [^{125}I]LRSC high-affinity binding under equilibrium and kinetic conditions (respectively 41, 40 and 30 nM) suggested that the same set of receptors mediates all three phenomena. These values are also close to the apparent K_D we determined for the stimulation of the synthesis of an apical membrane protein by type I collagen (~90 nM, Vega-Salas et al., 1987*b*). In order to test this hypothesis we studied the competition of LRSC, anti-laminin antibodies and anti-fibronectin antibodies in the medium with cell attachment to an agarose/330 nM collagen substrate (Table 2). At 37°C, 40 nM LRSC only caused a partial cell displacement whereas 330 nM LRSC resulted in a twofold increase, blocked by the addition of anti-laminin antibody. At 4°C, though, LRSC completely blocked the cell attachment. An anti-fibronectin antibody failed to produce any effect. These results are clearly compatible with a model in which a relatively small population of collagen receptors (~120,000/cell with a K_d of 30–40 nM) mediates the initial attachment of suspended MDCK cells to the substrate and triggers the exocytosis of laminin.

Partial Biochemical Characterization of Collagen Receptors

To investigate the nature of collagen receptors, we studied the effect of hydrolytic enzymes on LRSC binding and cell attachment to collagen. Trypsin was a very effective inhibitor in both assays (Table 3); the capacity for attachment was recovered 10–15 hr after trypsin treatment (Fig. 9), and this recovery was abolished by cycloheximide. Neuraminidase and chondroitinase ABC also had an effect, opening the possibility that these receptors are of a proteoglycan nature.

Rapid Recruitment of Collagen Receptors on the Substrate During Cell Attachment

Goldberg (1979) has shown the presence of LRSC receptors in fibroblasts but failed to find them in confluent MDCK cell monolayers. Because LRSC

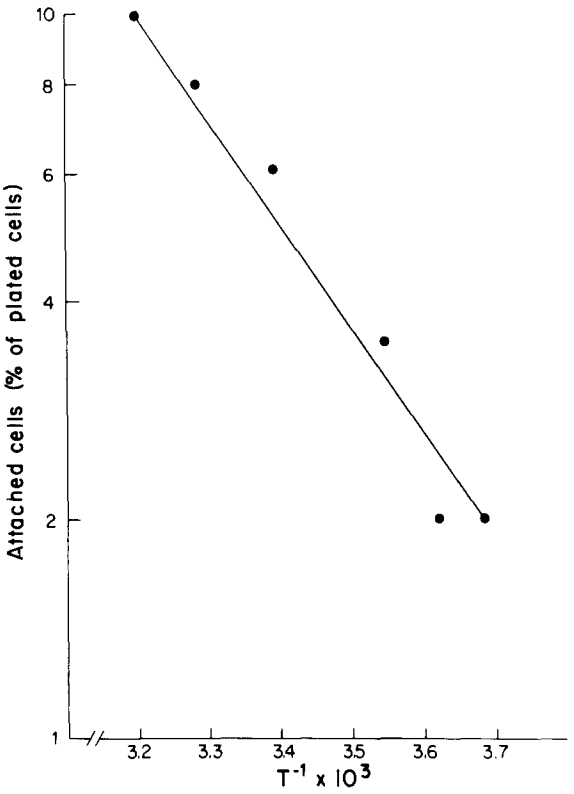

Fig. 8. Effect of temperature on the attachment of MDCK cells in suspension culture to a defined agarose/LRSC substrate. MDCK cells were processed as described above, but the cells were spun onto a substrate containing 3% agarose and 330 nM LRSC and incubated for 30 min at various temperatures. The loose cells were washed out with PBS and the attached cells counted. The data from two independent experiments are expressed as the logarithm of the number of cells/field that attached as a function of the inverse of absolute temperature (Arrhenius plot). The data fitted a linear regression under these coordinates with a regression coefficient = 0.92. The slope of this plot indicates an activation energy of 8.3 kcal/mol

receptors are present in suspended MDCK cells (this report), they may be cryptic confluent monolayers, e.g. on the basolateral domain, and therefore not accessible to LRSC added to the apical medium or they may simply disappear after attachment. To test these two possibilities, confluent MDCK and BHK (fibroblastic kidney cell) monolayers were grown to confluency on 2-μm pore nylon filter chambers. [^{125}I]LRSC was added either to the apical or to the basal medium. MDCK monolayers showed 7.1 times more LRSC binding to the basolateral than to the apical domain. BHK monolayers, despite their leakage (differently from MDCK monolayers, BHK had no transepithelial electrical resistance), showed the opposite polarity: they bound nine times more LRSC from the free side than MDCK (Table 4). The results with BHK cells serve as a control, indicating that the higher

Table 2. Inhibition of MDCK cell attachment by soluble collagen and antibodies

Free LRSC (nM)	LRSC in substrate (nM)	Antibody	Attached cells (% of control)
0	330	—	100
40	330	—	43 ± 16*
170	330	—	160 ± 8*
330	330	—	220 ± 52*
330	330	Anti-laminin	91 ± 14
0	330	Anti-laminin	125 ± 108
0	330	Anti-fibronectin	140 ± 10
0	0	—	1 ± 0.1*
Low temperature (4°C):			
0	330	—	6.1 ± 2.8*
30	330	—	2.7 ± 1.0*
300	330	—	1.0 ± 0.3*

Aliquots of a suspension culture of MDCK cells were incubated with various concentrations of LRSC for 30 min. Some aliquots were incubated in the presence of antibodies for 15 additional minutes. The cells were spun onto a substrate made of 330 nM LRSC in 3% agarose, and incubated at 37 or 4°C for 60 min. The data from three independent experiments are shown as per cent of the control value (top line: 100% corresponds to 29 ± 10% of total plated cells).
* Indicates the values significantly different ($P < 0.05$) from the control.

Table 3. Enzymatic sensitivity of LRSC binding and MDCK cell attachment

Enzyme	Concentration	LRSC binding (% of control)	Cell attachment (% of control)
Trypsin	0.5 mg/ml	18 ± 16*	42 ± 18*
Trypsin + Trypsin inhibitor	0.5 mg/ml 3.0 mg/ml	113 ± 5	127 ± 20
Hyaluronidase	1000 mg/ml	168 ± 57	124 ± 44
Neuraminidase	1.0 U/ml	84 ± 3*	54 ± 8*
Chondroitinase ABC	0.1 U/ml	61 ± 16*	42 ± 6*
Heparinase	0.5 U/ml	74 ± 27	97 ± 13
Heparitinase	0.5 U/ml	71 ± 6	89 ± 8

MDCK cells were kept in suspension culture for 12 hr. The cells were incubated at 37°C for 30 min (pH 7.3) in the presence of the enzymes. In the case of trypsin, 3.0 mg/ml of trypsin inhibitor was added after that period, while both were added together for the log "trypsin + trypsin inhibitor." [^{125}I]LRSC was added to some cell aliquots after the incubation with the enzymes, and incubated with the cells for 40 min ("Binding"). Some cell aliquots were spun onto a 3% agarose substrate containing 330 mM LRSC ("Cell attachment"). The LRSC binding and the cell attachment were measured as described above. The data from four independent experiments are expressed as % of control (no enzyme) value (control binding was 14,111 ± 4680 cpm/10^6 cells; control attachment was 12 ± 3% of plated cells). * Indicates values significantly different ($P < 0.05$) from the control value.

Table 4. Binding of [^{125}I]LRSC to confluent monolayers of cells grown on nylon filters

Cell type	Apical surface (%)	Basolateral surface (%)
MDCK (epithelial)	14	100
BHK (fibroblasts)	126	7

MDCK and BHK cells were grown to confluency on nylon filters (2 μm pore). The transepithelial electrical resistence (TER) was measured for each filter immediately before the experiment. Only filters showing TER over 50 Ω · cm^2 were used. The cells were incubated for 1 hr at 4°C (in order to avoid endocytosis) with [^{125}I]LRSC added either from the apical or the basolateral (filter) side. The cells were extensively washed for 2 hr with cold PBS and counted. Filters without cells were incubated and washed in the same conditions and the values subtracted (479 cpm); the results are expressed as percent of the average of cpm for MDCK with ligand from the basolateral surface (4169 cpm). Note: (i) that each filter contains ~1.5 × 10^5 cells at confluency, so that the level of binding to the basolateral surface of MDCK cells compares well with the binding to cells in suspension culture; and (ii) that most of the ligand binds to epithelial cells at the basolateral surface while most of the ligand binds to fibroblasts on the free surface. The low amount of binding to fibroblasts from the filter side rules out a possible nonspecific trapping in the filter.

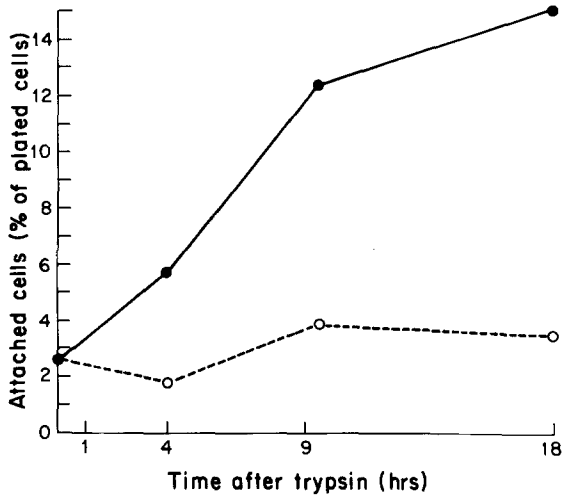

Fig. 9. Attachment of MDCK cells to a defined agarose/LRSC substrate at various times after dissociation. At time 0, confluent monolayers of MDCK cells were dissociated by EDTA-trypsin and resuspended for suspension culture in the presence (○) and in the absence (●) of 20 μg/ml cycloheximide. At various times aliquots of each suspension were spun onto a 3% agarose, 330 nM LRSC substrate. The cells were incubated for 60 min at 37°C, washed and counted. The data from two experiments are expressed as number of cells/field attached on the substrate. Note that cells recover their ability to attach to LRSC within 15 hr after dissociation and that this recovery is abolished by cycloheximide

binding of LRSC to the basolateral domain of MDCK cells is not due to simple LRSC trapping in the filter.

To test whether the high-affinity collagen receptors are recruited during attachment, we assayed the displacement of prebound [^{125}I]LRSC during cell attachment to a collagen substrate. MDCK cells in suspension culture were incubated in the presence of [^{125}I]LRSC and then quickly centrifuged through a dextran cushion (to eliminate the free ligand) onto a 330 nM LRSC-agarose substrate located in the bottom of the tube. The supernatant and the top of the dextran cushion were rapidly aspirated, and the cells were incubated for various times on the substrate. Prebound [^{125}I]LRSC was almost completely displaced (80%) in 12 min (Fig. 10). Control samples, treated identically except for the use of pure 3% agarose as a substrate, showed little displacement. This experiment suggests that LRSC receptors are recruited onto the attached surface of the plasma membrane within the first few minutes of attachment.

Discussion

The results of this report indicate that MDCK cells in suspension culture display a population (~1.2 × 10^5 sites per cell) of high affinity collagen binding sites (K_D 30–40 nM) involved in early cell attachment. Equilibrium LRSC binding data from live cells (Fig. 2) were useless for K_D determination because unspecific binding could not be measured. However, equilibrium binding data from formalin-fixed cells and kinetic binding data from live cells showed the same K_D values (Figs. 3 and 4). In addition, kinetic data indicated that both endocytosis of ligand and the appearance of a second population of LRSC-binding sites are responsible for the increase in LRSC binding at concentrations above 40 nM in live cells. The high-affinity collagen receptors appear to be carried by a molecule different from laminin because: (i) most laminin is intracellular in suspended MDCK cells (Figs. 5 and 6), (ii) they do not discriminate between type I and type IV collagens, as laminin does (Woodley et al., 1983), (iii) a polyclonal anti-laminin antibody does not affect early cell attachment (Table 2), and (iv) MDCK cells attach more efficiently to collagens than to extracellular laminin during short (<1 hr) incubations (Table 1). The collagen receptors are also different from fibronectin because: (i) there was no fibronectin immunoblot reactivity in MDCK cell extracts and MDCK cells attached poorly to fibronectin during short incubations (<1 hr) (Table 1), (ii) a polyclonal anti-fibronectin antibody failed to affect cell attachment (Table 3), and (iii) all experiments were

carried out in serum-free medium. The role of laminin receptors and fibronectin receptors during a later phase of MDCK cell attachment is shown by the ability of these cells to attach to either exogenous substrate after longer incubations.

The collagen receptors described here are quickly recruited on the substrate during cell attachment. Their occupancy either by soluble collagen or, presumably, by substrate collagen, results in the exocytosis of laminin, and perhaps other basement membrane components, from an intracellular pool. Work from three other laboratories has demonstrated the existence of a 65-kD laminin receptor (Lesot, Kuhl & von der Mark, 1982; Malinoff & Wicha, 1983; Rao et al., 1983; Terranova et al., 1983) and a fibronectin receptor (for a review *see* Ruoslahti & Piershbacher, 1986). An entire family of substrate receptors to extracellular matrix (ECM) has been recently identified (Yamada et al., 1985). These receptors may bind various ECM components (Horwitz et al., 1985). Although we show here no direct evidence for a laminin receptor in MDCK cells, it is supported by the observation that most of the exocytosed laminin remains associated with the cell surface (Fig. 5) and the cells attach very well to exogenous laminin during long (4 hr) incubations (Table 1). Since laminin binds to type I collagen (although with less affinity than to type IV, *see* Woodley et al., 1983), the kinetic data (Fig. 4) suggests that laminin (presumably bound to laminin receptors) mediates a secondary collagen binding mechanism which starts to operate 20 min after the first cell-collagen interaction. The surface appearance of laminin and laminin receptors would explain the late (4 hr) increase in the preference of the cells for laminin. Our work is in agreement with previous observations from other laboratories, such as the collagen requirement of early (1 hr) hepatocyte attachment (Rubin et al., 1978) and the laminin requirement of PAM 212 (epithelial) cells during longer (2–6 hr) incubations (Terranova et al., 1980). Carlin and coworkers (1983) have reported the induction of laminin and entactin synthesis and secretion by retinoic acid and dibutyryl cyclic AMP in F9 cells. The relationship between their result and the induction of laminin exocytosis by collagen shown here requires further investigation.

Collagen receptors have been described previously in other systems. Anchorins mediate the binding of chondrocytes to type II collagen (Mollenhauer & von der Mark, 1983; Mollenhauer et al., 1984). Chiang and coworkers (1978) and Goldberg (1979; 1982; Goldberg & Burgeson, 1982) demonstrated the presence of collagen receptors in fibroblasts. These receptors exhibit affinities three orders of magnitude higher for collagen ($K_D = 1.2 \times 10^{-11}$ M) than those described here for MDCK cells.

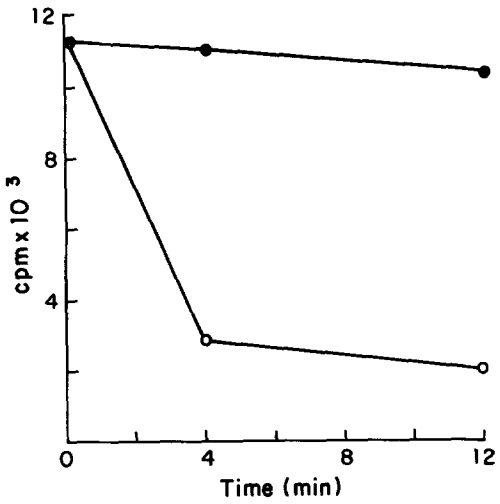

Fig. 10. Effect of the substrate on the dissociation of [^{125}I]LRSC from MDCK cells. The cells were kept overnight in suspension culture and incubated in the presence of ~10^6 cpm/ml iodinated LRSC for 40 min. The cells were spun through a 10% dextran cushion. At the bottom of the tubes there were agarose gels with (○) or without (●) 10% collagen. Immediately after centrifugation the supernatant with the free ligand was removed with the top of the dextran cushion in order to avoid diffusion of free ligand. The remaining portion of the cushion was aspirated at various times. The gels were removed along with the cells (and ~0.1 ml dextran solution) and counted. The means from three independent experiments are shown. Note that [^{125}I]LRSC dissociates much faster when the cells are spun onto a collagen-containing gel: mean time of dissociation calculated by fitting the data with an exponential function by the least-squares method is six min for collagen-agarose gels and 165 min for pure agarose gels

In epithelial cells, collagen binding sites have been demonstrated directly only in hepatocytes (Rubin et al., 1978), and recently they have been isolated (Rubin et al., 1986). Bernfield and coworkers (Koda, Rapraeger & Bernfield, 1985; Rapraeger et al., 1985) have reported a membrane proteoglycan that binds to interstitial collagens in mammary cells. Functional evidence for the existence of collagen receptors has been provided by the work of Sugrue and Hay. These authors have shown that soluble extracellular matrix components such as types I and IV collagen, laminin or fibronectin have a dramatic effect on the structure of the basal plasmalemma and cytoskeleton of corneal epithelium (Sugrue & Hay, 1981) and that laminin and collagen appear to interact directly with the cell (Sugrue & Hay, 1982). Laminin and fibronectin stimulate the production of collagen to twice the control levels (Sugrue & Hay, 1986). These results highlight the existence of separate, but not necessarily independent, mechanisms of interaction between epithelial cells and the substrate. We speculate that collagen receptors might play a role in vivo in two different circumstances: (i)

during development, when basement membranes are yet inexistent or very thin (Bernfield & Banerjee, 1978), or (ii) anchoring epithelial cells directly to type IV collagen in basement membranes, since we found no major differences in the recognition of type I and type IV collagens by MDCK cells.

The attachment to the substrate is an essential step in the development of surface polarity by epithelial cells (Rodriguez-Boulan, 1983*a*). 4–6 hours after attachment, the polarity of the cell manifests itself in the asymmetric budding of viruses from the cell surface (Rodriguez-Boulan et al., 1983). Intrinsic surface markers show different behaviors: while some basolateral markers progressively polarize as MDCK cells establish contact with the substrate and with other cells and develop intercellular junctions (Balcarova-Stander et al., 1984; Herzlinger & Ojakian, 1984), certain apical markers are polarized even in subconfluent monolayers in the absence of tight junctions (Vega-Salas, Salas & Rodriguez-Boulan, 1985; Vega-Salas et al., 1987*a*). The results of this report indicate that, during attachment, collagen receptors become recruited on the adherent surface and are basal in confluent monolayers. Unpublished results (P.J.I. Salas, D.E. Vega-Salas & E. Rodriguez-Boulan) indicate that the exocytosis of laminin in response to collagen binding occurs towards the free surface of attaching MDCK cells, although in confluent monolayers laminin has the same basal location described in other epithelial tissues (Timpl et al., 1979). Sugrue and Hay (1986) have recently shown that laminin, fibronectin and collagen binding sites are found on the basal surface of the corneal epithelium. Nitsch and Wollman (1980) and Chambard et al. (1981) have made the intriguing observation that the polarization of thyroid cells is reversed in response to the addition of collagen or serum to the medium. Hall and coworkers (1982) reported a similar effect of collagen on MDCK cells. Thus, extensive rearrangements of molecules binding to extracellular matrix components occur during cell attachment; these rearrangements lead to the establishment of polarity following a still unknown program. The characterization of the early cell-cell and cell-substrate interactions and of the molecules involved should provide a basis for the elucidation of this program and is currently under investigation in our laboratory.

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