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Differential effects of the cytoplasmic domains of cell adhesion molecules on cell aggregation and sorting-out

(morphogenesis/pattern formation/cytoskeleton/neural cell adhesion molecule/liver cell adhesion molecule)

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ABSTRACT **Cell adhesion molecules (CAMs) are cell surface glycoproteins that play important roles in morphogenesis and histogenesis, particularly in defining discrete borders between cell populations. Previous studies have suggested that the cytoplasmic domains of CAMs play a significant role in their adhesion properties. These domains may also be involved in regulating other cellular interactions, such as those involved in the sorting-out of cells to form tissues. In the present studies, we have compared the effects of replacing the cytoplasmic domain of one CAM with that of another CAM of different homophilic binding specificity on cell adhesion and cell sorting-out. The molecules studied were liver CAM (L-CAM) and the neural CAM (N-CAM) sd polypeptide. One cDNA was constructed that encodes a chimeric molecule composed of the extracellular domain of L-CAM and the cytoplasmic plus transmembrane domains of the sd polypeptide of chicken N-CAM (called L/N-CAM). Another was constructed encoding a truncated L-CAM missing the last 50 residues of the cytoplasmic domain. Permanently transfected lines of mouse L cells were obtained expressing the truncated L-CAM ("L-L-50 cells") or the chimeric L/N-CAM ("L-L/N cells") and were compared with cells expressing intact L-CAM ("L-L cells"). Immunoblotting and ELISA analyses demonstrated that these various cell lines expressed similar amounts of CAMs at the cell surface. Aggregation of L-L and L-L/N cells occurred at similar rates in short-term aggregation assays and was inhibited by antibodies to the extracellular L-CAM binding domain. In contrast, L-L-50 cells did not aggregate. Incubation of transfected cells with cytochalasin D, which disrupts microfilaments, markedly inhibited aggregation of L-L cells but had no effect on L-L/N cell aggregation. Mixed L-L and L-L/N cells co-aggregated in short-term assays; in the longer-term sorting-out assays, however, they behaved differently: L-L cells sorted out from both L-L/N and untransfected cells, whereas L-L/N cells did not sort out from untransfected cells. These studies not only suggest that interactions of cytoplasmic domains of different CAMs with the cytoskeleton can modulate cell adhesion but also suggest that specific interactions with certain cytoskeletal components are required for events such as cell sorting and cell patterning.**

Selectivity in the adhesion of cells is believed to play a fundamental role in organizing tissues with multiple cell types. Different types of animal cells, after dissociation, have the ability to sort out from one another when mixed (1–3). Although a number of hypotheses have been proposed to explain the mechanism of cell sorting, its molecular basis is just beginning to be elucidated. Among the key elements in this process are cell–cell interactions mediated by cell adhesion molecules (CAMs) (4, 5).

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CAMs are cell surface glycoproteins that have been variously categorized; one useful criterion is whether or not Ca^{2+} is required for their binding activity. CAMs that do not require Ca^{2+} for binding include neural CAM (N-CAM) (6) and neuron–glia CAM (Ng-CAM) (7), whereas liver CAM (L-CAM) or uvomorulin (8–10), adherens junction-specific CAM (A-CAM) (11) or N-cadherin (12), and other cadherins (12) are examples of CAMs that are Ca^{2+} dependent. Transfection of cDNAs of both categories of CAMs into cells that normally do not express these molecules has demonstrated their direct involvement in cell adhesion (13, 14).

It has been proposed that cell surface modulation involving the cytoskeleton plays important roles in cell adhesion and transmembrane signaling (15). Studies (16) utilizing cells transfected with CAMs support this view and suggest the importance of the CAM cytoplasmic domain in cell adhesion. This domain interacts with specific intracellular components, and such interactions can be disrupted by deletion of the 37 carboxyl-terminal amino acids of L-CAM (17, 18).

It is of particular relevance that cells expressing various CAMs tend to sort out from one another to form collectives linked by a particular CAM. This cell segregation is dependent both on the specificity and the amount of each CAM expressed at the cell surface, as shown by the ability of mouse S180 cells expressing L-CAM or N-cadherin (19), and L-cells expressing either E- or P-cadherin (20), to sort out from each other. Cell sorting also must depend upon interactions of particular CAM cytoplasmic domains with the cytoskeleton (19, 21), interactions that affect and are affected by changes in cell shape and movement.

To investigate further such mechanisms as they might be reflected in CAM-mediated cell aggregation and cell sorting, cDNA clones encoding L-CAM, the extracellular domain of L-CAM linked to the cytoplasmic domain of N-CAM (sd form) (13) (L/N-CAM), or a truncated L-CAM lacking the last 50 residues of the carboxyl terminus, were each transfected into mouse L cells. Cells expressing the truncated L-CAM ("L-L-50 cells") failed to aggregate, in agreement with previous reports (16). Both cells expressing intact L-CAM ("L-L cells") and those expressing L/N-CAM ("L-L/N cells") expressed high levels of CAMs at the cell surface and aggregated well with themselves and with each other in short-term aggregation assays. Microfilament disruption with cytochalasin D inhibited the aggregation of L-L cells but had no effect on L-L/N cells. In culture, L-L cells sorted out from both untransfected cells and L-L/N cells, whereas L-L/N cells did not sort out from untransfected cells. These results support the conclusion that differential interactions of the cytoplasmic domains of different CAMs with cytoskeletal elements can affect morphogenetic events subsequent to specific homophilic CAM binding.

Abbreviations: CAM, cell adhesion molecule; N-CAM, neural CAM; L-CAM, liver CAM.

MATERIALS AND METHODS

DNA Constructs. All DNA constructs were prepared with cDNA clones for chicken N-CAM (13, 22) and L-CAM (13, 23, 24). DNA modification and restriction enzymes were purchased from Pharmacia. The L-CAM construct used was previously described (24). For the synthesis of the L/N chimeric construct, N-CAM (sd) cDNA in pGEM (pEC1A1) (25, 26) was digested with *Nco* I and filled with the Klenow fragment of DNA polymerase. *Bam*HI linkers were then blunt-end ligated onto both ends of the plasmid, which was then digested with *Stu* I and *Bam*HI to yield a 430-base-pair fragment containing the coding sequences for the transmembrane and cytoplasmic domains of N-CAM (sd) from amino acid residues 690–811 (22). The L-CAM plasmid pEC350 (24) was digested with *Kpn* I. The *Kpn* I–*Kpn* I fragment was removed and stored and the remaining plasmid was religated to yield pEC350ΔK. pEC350ΔK was then digested with *Nco* I, treated with mung bean nuclease to remove single-stranded DNA ("polished"), and subsequently digested with *Bam*HI to yield pEC350ΔKΔNB. The *Stu* I–*Bam*HI fragment from the N-CAM plasmid was then ligated into the *Nco* I–*Bam*HI site of pEC350ΔNB to yield pLΔKNsd. The *Kpn* I–*Kpn* I L-CAM fragment of pEC350 was ligated into the *Kpn* I site of pLΔKNsd to yield plasmid pLNsd, which codes for 495 amino acids of the extracellular domain of L-CAM and the cytoplasmic plus transmembrane domains of the N-CAM sd polypeptide. This junction was sequenced and shown to contain the correct sequences coding for Leu-Ala 494–495 of L-CAM and Leu-Gly 690–691 of N-CAM. To synthesize an expression vector suitable for transfection, the plasmid was digested with *Eco*RI and the ends were filled by using Klenow fragment. *Bam*HI linkers were ligated onto both ends of the plasmid, which was then cut with *Bam*HI. The resulting fragment was purified and inserted into the *Bgl* II site of the mammalian expression vector pKSV10 (24).

The deleted L-CAM cDNA was constructed by digesting pEC350 with *Sph* I and polishing with mung bean nuclease. The resulting plasmid was religated, cut with *Xba* I, and polished. *Sph* I linkers were then ligated in the plasmid at the former *Xba* I site. After religation, multiple termination codons were inserted into the *Hin*cII site of the plasmid. The plasmid was then digested with *Bam*HI and *Sph* I, followed by exonuclease III, to yield a plasmid encoding L-50, a truncated L-CAM missing 50 amino acids from its cytoplasmic domain and including three amino acids from the vector sequence as verified by DNA sequencing. The fragment containing the deleted L-CAM was then ligated into pKSV10 as described above.

Cell Culture and Transfection. All transfections were performed with the calcium phosphate protocol on mouse L-M(TK$^-$) cells (American Type Culture Collection CCL 1.3) as described (13).

Immunoblotting and Immunofluorescence Staining. Detection of CAM cell surface expression by immunofluorescence staining was carried out as described (13). For quantitation of surface expression, transfected cells were plated in 96-well tissue culture plates (Falcon), fixed, incubated with polyclonal anti-L-CAM antibodies for 1 hr, and quantitated by using a Vectastain ELISA kit (Vector Laboratories). Immunoblotting was performed as described (27).

Cell Aggregation and Sorting-Out Assays. Cells were assayed for aggregation and co-aggregation (13, 20) and sorting-out as described (19). Prior to both assays, cells were incubated with 10 mM sodium butyrate (Sigma) in culture medium for 6 hr.

RESULTS

Synthesis and Expression of CAMs. A schematic diagram of the L-CAM, L/N-CAM, and L-50 constructs is presented in Fig. 1. Immunoblot analysis with specific antibodies to

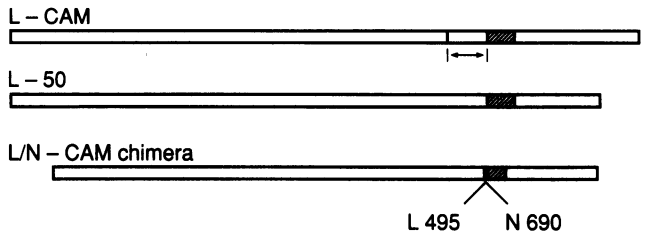

FIG. 1. Schematic diagram of L-CAM, L-50, and L/N-CAM. All three molecules have identical extracellular domains with the exception of a 45 amino acid segment, denoted by an arrow in the L-CAM construct, which is missing in the chimeric molecule. Hatched areas represent transmembrane domains. The junction between L-CAM and N-CAM amino acid sequences is indicated in the figure, with amino acids numbered as described (22, 23).

L-CAM showed that permanently transfected L-L cells (Fig. 2*A*, lane 1), L-L/N cells (Fig. 2*A*, lane 2), or L-L-50 cells (Fig. 2*A*, lane 3) expressed the transfected CAM with molecular weights appropriate for the cDNA constructs. These cell lines expressed their respective gene products on the cell surface as shown by immunostaining with polyclonal anti-L-CAM antibodies (Fig. 2 *B–E*).

Quantitation of Cell Aggregation. The amount of L-CAM and L/N-CAM at the cell surface was determined with an enzyme-linked immunosorbant assay (ELISA) on cells in monolayer culture. The level of L-CAM detected on both

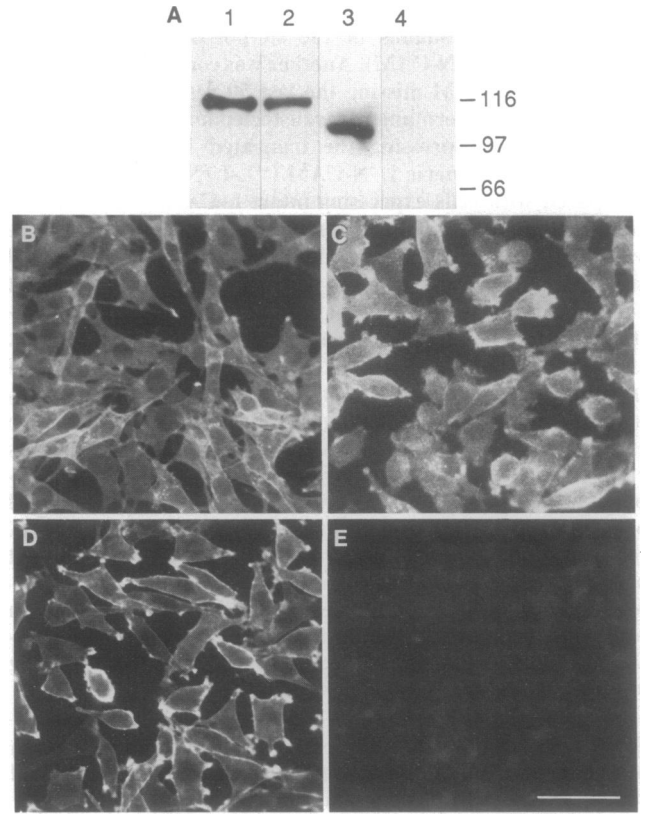

FIG. 2. Expression of transfected CAMs. (*A*) Immunoblots. Extracts of L-L cells (lane 1), L-L/N cells (lane 2), membranes of L-L-50 cells (lane 3), and untransfected L cells (lane 4) were resolved by NaDodSO$_4$/PAGE and visualized with polyclonal antibodies to chicken L-CAM (9). Molecular weights of standard proteins $\times 10^{-3}$ are shown on the right. (*B–E*) Cell surface expression of CAMs. Fluorescence micrographs of L-L (*B*), L-L-50 (*C*), and L-L/N cells (*D* and *E*) induced with 10 mM butyrate (12 hr) and stained with rabbit polyclonal antibodies to chicken L-CAM (*B–D*) or to chicken N-CAM (*E*) (13). (Bar = 50 μm.)

L-L and L-L/N cells was similar whether the cell number (not shown) or antibody concentration (Fig. 3*A*) was varied, demonstrating that similar quantities of CAM were present at the surface of each of these cell lines.

CAM-mediated aggregation of transfected cells was determined by measuring the disappearance of single cells and its inhibition by polyclonal anti-L-CAM Fab' fragments. Aggregation of L-L and L-L/N cells occurred at similar rates and to the same extent (0.85; Fig. 3*B*) and was inhibited (by 60%) by anti-L-CAM Fab' fragments. Chelation of Ca^{2+} by addition of EGTA also inhibited aggregation of both cell lines (not shown). The baseline level of aggregation of L-L-50 cells and untransfected cells was low after a 90-min incubation and was not inhibitable by anti-L-CAM Fab' fragments (Fig. 3*B*). In agreement with previous results (13), N-CAM sd transfectants (L-N cells) aggregated, and this aggregation was completely inhibited by N-CAM antibody fragments (Table 1).

The possibility of differential interaction of cytoplasmic domains with components of the cytoskeleton was also investigated. L-L and L-L/N cells were incubated with the microfilament-disrupted drug cytochalasin D or the microtubule-disrupting drug nocodazole for 15 min at 4°C and then assayed for binding activity (Table 1). Maximum inhibition of L-L cell aggregation was observed at a final drug concentration of 1 μg/ml (not shown). At this concentration of cytochalasin D, L-L cells aggregated to a level only 51% that of untreated cells; no effect on the aggregation of L-L/N cells or L-N cells was observed. In contrast, nocadazole at 1 μg/ml, which disrupts microtubules, affected the aggregation of L-L/N cells and L-N cells, although to a lesser extent, but had no effect on L-L cell aggregation. In immunoprecipitation experiments, L-CAM coprecipitated with a 100-kDa intracellular component known to interact with the cytoplasmic domain of L-CAM (17, 18) only in the L-L cells, and not in either the L-L/N cells or the L-N cells (not shown). Taken together, these findings suggest that the cytoplasmic domain (sd) of N-CAM has a different relation, if any, to the actin cytoskeleton than does L-CAM.

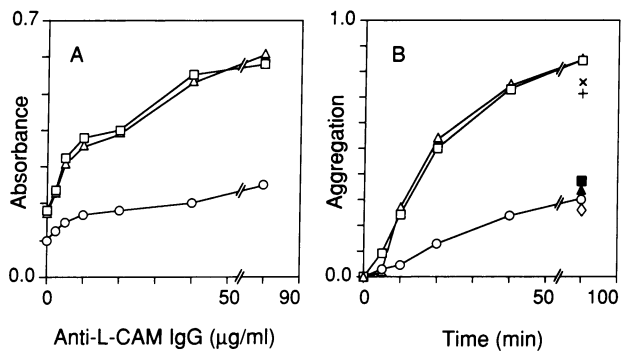

FIG. 3. Aggregation of transfected cell lines. (*A*) Quantitation of L-CAM and L/N-CAM cell surface expression. L-L (△), L-L/N (□), and untransfected (○) cells were incubated with increasing concentrations of anti-L-CAM antibody (abscissa) for 1 hr and washed with phosphate-buffered saline (PBS; 1.5 mM KH_2PO_4/2.7 mM KCl/4.3 mM Na_2HPO_4/137 mM NaCl). The amount of antibody remaining was subsequently determined by utilizing a biotinylated horseradish peroxidase system (Vectastain). Ordinate is the absorbance at 410 nm. Points represent means (n = 3, SEM < 5%). (*B*) Rates of aggregation. Cells were released from the culture dish and assayed for aggregation by measuring the disappearance of single cells (24). Aggregation is defined by $(N_0 - N_t)/N_0$, where N_0 and N_t = the number of single cells at time 0 and time t, respectively. Both L-L (△) and L-L/N (□) cells have similar aggregation rates which are significantly higher than the rate of the control cells (○). Anti-L-CAM Fab' fragments inhibited the binding of both L-L (▲) and L-L/N (■) cells, while anti-N-CAM Fab' fragments had little effect on L-L (×) and L-L/N (+) cell aggregation. L-L-50 cells did not aggregate even after 90 min (◇). Points represent means (n = 2, mean deviations < 5%).

Table 1. Effects on cell aggregation of cytoskeleton-disrupting drugs

Treatment	Aggregation, % L-L cells	L-L/N cells	L-N cells
None	100	100	100*
Fab'	38 ± 4	35 ± 6	5 ± 2
Cytochalasin D	51 ± 5	98 ± 3	96 ± 3
Nocodazole	95 ± 4	75 ± 6	77 ± 5

Numbers are means ± mean deviations (n = 2) and represent the percentage of aggregation relative to untreated cells. (See legend to Fig. 3 for details of aggregation assays.)
*Aggregation by L-N cells was less extensive (30% over untransfected cells) than that of either L-L or L-L/N cells (80%).

Differential Properties of CAM Cytoplasmic Domains in Cell Sorting-Out. In order to examine the sorting properties of L-L and L-L/N cells in terms of differential properties of their cytoplasmic domains, it was essential to determine whether they could bind to each other. To determine that the two transfected lines could interact with one another, aggregation assays were performed on mixed cell populations each labeled with a different vital dye (Fig. 4). Cells in clusters of 20–50 cells were counted and scored for the percentage of cells labeled with each dye, and results are plotted as histograms of the number of clusters with a given percentage of labeled cells. L-L/N and L-L cells aggregated to form heterogeneous cell clusters composed of approximately equal numbers of both cell types (compare Fig. 5 *A* and *B*). When L-L or L-L/N cells were mixed with untransfected cells, however, aggregates containing primarily one cell type were observed (Fig. 4 *C* and *D*).

Cell sorting depends on factors in addition to cell–cell binding (28) and, to the extent that motion and process

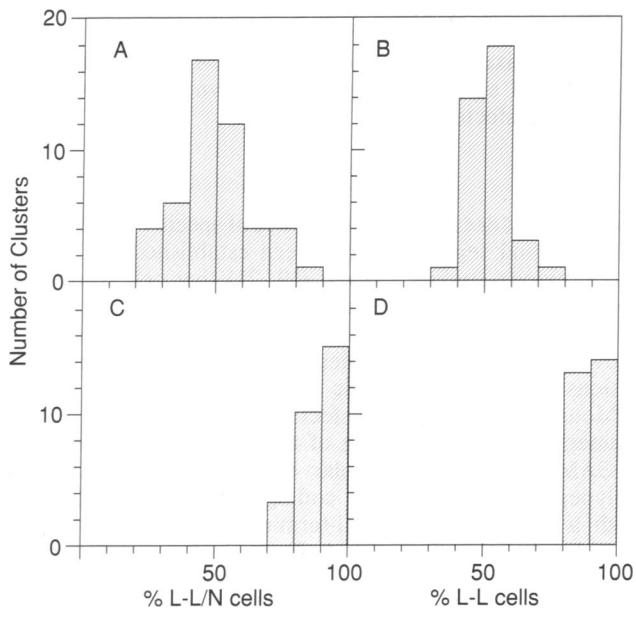

FIG. 4. Quantitation analysis of co-aggregation of L-L and L-L/N cells. L-L, L-L/N, and untransfected cells were differentially labeled with the fluorescent carbocyanine dyes dil and diO (Molecular Probes), mixed pairwise at a 1:1 ratio, and allowed to aggregate for 30 min as described (19, 24). Aggregates of 20–50 cells were randomly selected, and the percentage of the indicated cell line was determined by counting the number of labeled cells of a particular color in each cluster. The L-L and L-L/N cells (*A*) co-aggregated to a similar extent as two populations of differentially labeled L-L cells (*B*). Little co-aggregation was observed when L-L/N or L-L cells were mixed with untransfected cells (*C* and *D*, respectively).

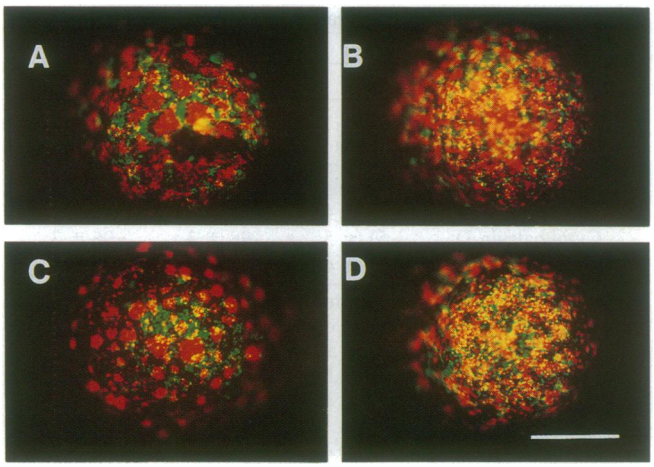

FIG. 5. Micrographs of cells in sorting-out assays. Cells were labeled with the fluorescent dyes dil and diO and assayed for sorting-out as described (19). Yellow arises from the superposition of red and green unsegregated cells. L-L cells (red) sorted out from the untransfected (green) control cells (*A*). In the presence of 250 μg of polyclonal anti-L-CAM, sorting-out of these two cells was inhibited (*B*). L-L cells (red) also sort out from L-L/N cells (green) (*C*). When untransfected (red) and L-L/N cells (green) were mixed, there was little sorting-out (*D*). (Bar = 500 μm.)

extension are involved, is expected to involve interactions between cytoskeletal elements and cytoplasmic domains of CAMs. We therefore determined whether L-L cells could sort out from L-L/N cells, even though both cell lines express adhesion molecules with similar extracellular domains and show similar aggregation properties. As previously observed for S180 cells (19), after an incubation period of 16 hr, mixed populations of L-L and untransfected cells sorted out (Fig. 5*A*), and this sorting-out was specifically inhibited by anti-L-CAM antibodies (Fig. 5*B*). Sorting-out of L-L from L-L/N cells was also observed when these two cell populations were mixed (Fig. 5*C*). In contrast, L-L/N cells (Fig. 5*D*) and N-CAM sd transfectants (not shown) did not sort out from untransfected cells.

DISCUSSION

Over the past decade, the importance of specific CAMs in cell adhesion and segregation has been established (4, 12, 29). Our previous studies showed (19, 21) that cell sorting-out depends upon both the specificity and the amount of a CAM expressed at the cell surface. The studies presented here show that sorting-out also depends upon the type and interactions of CAM cytoplasmic domains.

Mouse L cells were transfected with a cDNA encoding a chimeric protein consisting of the extracellular domain of L-CAM and the intracellular domain of the N-CAM sd polypeptide, with a cDNA coding for a truncated L-CAM with a 50-residue carboxyl-terminal deletion, and with a cDNA for authentic L-CAM. Permanently transfected cell lines expressing authentic L-CAM aggregated well, as previously shown (13, 24), whereas cells expressing the L-CAM deletion mutant did not aggregate, consistent with previous findings of others (16). L-L and L-L/N cells expressing similar levels of CAM at their surfaces had similar aggregation rates and co-aggregated with one another, but they had markedly different properties in cell sorting assays: L-L cells sorted out from L-L/N and untransfected cells, whereas L-L/N cells did not sort out from the untransfected cells. The behavior of L-L/N cells resembled that of N-CAM sd transfectants, which also aggregated but did not sort out from untransfected cells.

It is likely that the close collection and stabilization of a relatively large number of CAM molecules at a given part of the cell surface by means of various cytoskeletal attachments are necessary for effective cell adhesion. The observations that cytochalasin D inhibits the aggregation of L-L cells but not of L-L/N cells, and that nocodazole inhibits the aggregation of L-L/N cells but not of L-L cells, support this conclusion and suggest that the L-CAM and L/N-CAM cytoplasmic domains interact with different cytoskeletal components. In addition, the fact that aggregation fails when the L-CAM cytoplasmic domain is truncated but not when it is replaced by the cytoplasmic domain of an unrelated CAM indicates that the CAM intracellular domain plays a necessary permissive role in cell adhesion. The ability of L-L/N cells to co-aggregate with L-L cells supports the conclusion that the *specificity* of CAM binding (but not necessarily of subsequent cellular behavior) is determined by the extracellular domain.

An important issue arising from this and previous studies (19, 20) concerns the mechanisms involved in the differential cell sorting properties of L-L and L-L/N cells, inasmuch as both expressed similar levels of the L-CAM extracellular domain on the cell surface and aggregated at similar rates. During both cell aggregation and cell sorting, populations of cells come in contact and interact with one another through specific cell surface molecules. However, cell sorting-out also involves cell migration (28) and process extension, which, in the assays used here for cell aggregation, are not as important since cells are mechanically driven together.

It is clear that binding and subsequent cell sorting-out depend upon a variety of factors (Fig. 6) including (*i*) CAM specificity, (*ii*) CAM surface concentration, (*iii*) freedom of hinge regions (30) in places where the cell membrane is

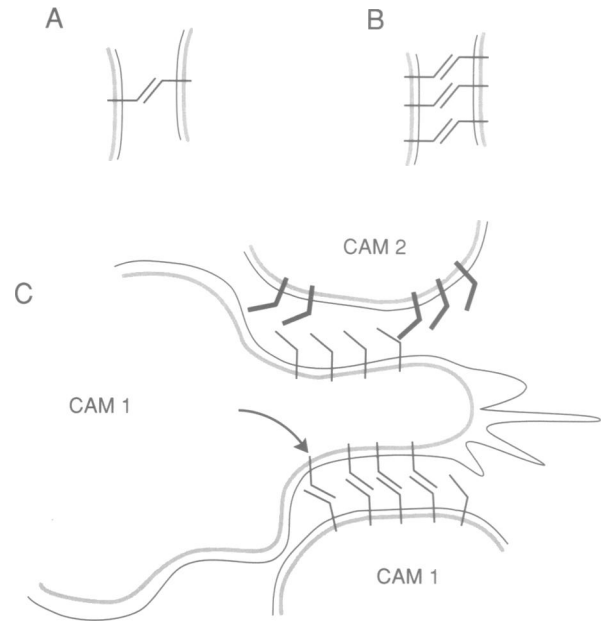

FIG. 6. Schematic of some postulated processes in cell adhesion followed by cell sorting. Process *A* represents an *initial* CAM–CAM binding event, which depends primarily on specificity and hinge properties in the extracellular binding domain. Process *B* suggests that CAM binding leading to cell adhesion requires the organization of CAM ligands at the cell surface. This is mediated by cytoskeletal interactions (cytoskeleton is schematized by the thick gray line beneath the cell membrane), resulting in stabilization of binding. Process *C* exemplifies the more complicated linking of CAM interactions (arrow) with cell movement and process extension. Combinations of these events lead to cell sorting-out and are strongly dependent upon specific interactions of CAM cytoplasmic domains with particular cytoskeletal elements.

rapidly changing shape, (*iv*) the type of cytoplasmic domain and its specific interaction with particular cytoskeletal components, as shown here, and (*v*) the readjustment of these components as process extension and movement occur. Further studies of cell movement and process extension *in vitro* may help to clarify the contribution of the last factor.

The processes illustrated in Fig. 6 may be altered by biochemical signals resulting from cell interactions themselves. These may generate specific recognition signals (e.g., second messengers) that result in a reorganization (15) of the cytoskeletal network. It is well established that the organization of cytoskeletal components is quite different in stationary cells and migrating cells (31–33). Cytoskeletal changes may in turn result in the redistribution of cell surface adhesion proteins facilitating adhesion, as has been previously postulated to occur with various CAMs (15) and substrate adhesion molecules (SAMs) (34). The observation that L-L/N cells aggregate as well as L-L cells suggests that processes *A* and *B* in Fig. 6 occur appropriately with either cytoplasmic domain. Thus while a binding structure composed of multiple CAMs of a given type of the cell surface is required for cell adhesion, more than one kind of interaction with cytoskeletal components can sustain such a structure. On the other hand, cell sorting-out requires a more specific interaction with selected cytoskeletal components. Whether process *C* fails to occur for L-L/N cells because the sd domains are linked to different cytoskeletal elements than the L-CAM domains (as suggested by the cytochalasin and nocodazole experiments) or whether there is in addition a difference in signaling events remains to be explored. Whatever the case, the concordant results observed with L-L/N cells and N-CAM-expressing cells in sorting-out experiments strongly suggest that cytoplasmic domains of CAMs play as important a role in tissue formation as their binding specificities.

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