

# Homophilic Adhesion between Ig Superfamily Carcinoembryonic Antigen Molecules Involves Double Reciprocal Bonds

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**Abstract.** Both carcinoembryonic antigen (CEA) and neural cell adhesion molecule (NCAM) belong to the immunoglobulin supergene family and have been demonstrated to function as homotypic  $\text{Ca}^{++}$ -independent intercellular adhesion molecules. CEA and NCAM cannot associate heterotypically indicating that they have different binding specificities. To define the domains of CEA involved in homotypic interaction, hybrid cDNAs consisting of various domains from CEA and NCAM were constructed and were transfected into a CHO-derived cell line; stable transfectant clones showing cell surface expression of CEA/NCAM chimeric-proteins were assessed for their adhesive properties by homotypic and heterotypic aggregation assays. The results indicate that all five of the Ig(C)-like domains of NCAM are required for intercellular adhesion while the COOH-terminal domain containing the fibronectin-like repeats is dispensable.

The results also show that adhesion mediated by CEA involves binding between the Ig(V)-like amino-terminal domain and one of the Ig(C)-like internal repeat domains: thus while transfectants expressing constructs containing either the N domain or the internal domains alone were incapable of homotypic adhesion, they formed heterotypic aggregates when mixed. Furthermore, peptides consisting of both the N domain and the third internal repeat domain of CEA blocked CEA-mediated cell aggregation, thus providing direct evidence for the involvement of the two domains in adhesion. We therefore propose a novel model for interactions between immunoglobulin supergene family members in which especially strong binding is effected by double reciprocal interactions between the V-like domains and C-like domains of antiparallel CEA molecules on apposing cell surfaces.

CELL-cell interactions mediated by intercellular adhesion molecules (CAMs)<sup>1</sup> are essential for numerous cellular responses, ranging from cell-mediated immune responses to the sorting of heterogeneous cell populations into organized arrays during tissue formation in organogenesis (Armstrong, 1989). A major proportion of known CAMs to date belong to the Ig gene superfamily (Williams and Barclay, 1988). This family of molecules is involved in many different types of cell surface recognition events, including specific intercellular adhesion (Edelman, 1985). Common to these molecules is the Ig homology unit which consists of two  $\beta$  sheets stabilized by disulfide bonds. Based on primary sequence similarities and secondary structure predictions, three homology unit types have been

defined: the variable like-domain (V-set) and two constant like-domains (C1-set or C2-set) (Williams and Barclay, 1988). Combinations of these sets are used in individual molecules to achieve binding functions with a variety of specificities.

Neural cell adhesion molecule (NCAM) is an Ig superfamily member involved chiefly in intercellular adhesion by  $\text{Ca}^{++}$ -independent, homotypic interactions (Cunningham et al., 1987; Rutishauser et al., 1988). This cell surface sialoglycoprotein is expressed in a wide variety of tissues and cells including neurons, muscle and glial cells, and plays important roles in tissue formation during embryogenesis and development (Edelman, 1984; Klein et al., 1988; Rutishauser and Jessell, 1988). NCAM has also recently been shown to be involved in signaling by activation of second messengers (Doherty et al., 1991; Frei et al., 1992). There are as many as 192 different isoforms of NCAM due to alternative splicing (Reyes et al., 1991; Barthels et al., 1992): the mouse NCAM-120 studied here consists of a 19-amino acid signal peptide, five similar C2-set domains of  $\sim 100$  amino acids each, followed by two partial fibronectin repeat domains and a COOH-terminal domain processed to give a

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1. *Abbreviations used in this paper:* CAMs, intercellular adhesion molecules; CKS, CMP-KDO synthetase; GPI, glyco-phosphatidylinositol; GPI-PLC, glycoposphatidylinositol phospholipase C; NCAM, neural cell adhesion molecule; TFMS, trifluoromethanesulfonic acid.

glyco-phosphatidylinositol (GPI) linkage to the external cell membrane (Barthels et al., 1987; Santoni et al., 1989).

CEA, another Ig superfamily member, belongs to a subfamily of closely related cell surface glycoproteins which are found in various human tumors as well as in normal tissues (Thompson et al., 1991). The protein and mRNA levels of CEA have been found to be higher in colon tumors relative to their normal adjacent tissues (Boucher et al., 1989). The appearance of CEA in the blood of patients bearing various types of cancers represents the basis for its widespread use as a clinical tumor marker in the management of cancer (Shively and Beatty, 1985). CEA has been demonstrated to function *in vitro* as a  $\text{Ca}^{++}$ -independent homotypic CAM (Benchimol et al., 1989). Ectopic production of CEA has been recently shown to perturb normal functions maintained by other CAMs such as E-cadherin and to block differentiation in certain cell types (Eidelman et al., 1993). The structure of CEA consists of a 34-amino acid processed leader sequence, a 108-amino acid V-set  $\text{NH}_2$ -terminal domain, three very similar pairs of C2-set internal domains of 178 amino acids each, and a 27-amino acid hydrophobic COOH-terminal domain (Beauchemin et al., 1987) which is processed to give a GPI-type membrane anchor (Takami et al., 1988; Hefta et al., 1988). The internal domains, each consisting of an A and B C2-set subdomain, are 80–82% homologous at the nucleotide sequence level and 67–70% at the amino acid sequence level, with the first 67 amino acids of each A subdomain showing 89–96% nucleotide sequence identity. CEA is highly glycosylated, bearing some 28 potential sites for Asn-linked glycosylation (Beauchemin et al., 1987).

The novel biological effects of CEA alluded to above have prompted us to investigate the molecular mechanism of homotypic cellular adhesion mediated by this glycoprotein. Previous studies have revealed that CEA transfectant cells will aggregate only with themselves and not with the parental nontransfectant cells (Benchimol et al., 1989; Zhou et al., 1990) and that direct and specific binding of CEA-expressing cell lines can be demonstrated to occur with immobilized purified CEA (Hostetter et al., 1990a,b; Levin and Griffin, 1991); this evidence, and results presented here, indicate that homotypic cellular adhesion is mediated by direct homophilic molecular interactions between CEA molecules on apposing cell surfaces. It has been reported recently that the  $\text{NH}_2$ -terminal domain of CEA is necessary but not sufficient for CEA-mediated adhesion (Oikawa et al., 1991). To identify the nature of this homotypic adhesion, we made use of our previous observation that the two Ig superfamily CAMs, CEA and NCAM, have distinct binding specificities (Zhou et al., 1990). Hybrid CEA-NCAM cDNAs were constructed and introduced into CHO-derived LR-73 cells (Pollard and Stanners, 1979) to determine adhesion specificities. Our results demonstrate that both the V-like domain and a C-like domain of CEA are involved, but that either domain alone is completely ineffective in mediating homotypic intercellular adhesion; both types of domains are required in a domain-heterophilic interaction between CEA molecules on apposing cell surfaces. We propose a model where binding occurs between the V-like domain and the C-like domain of anti-parallel CEA molecules in a double-reciprocal symmetrical fashion.

## Materials and Methods

### CEA/NCAM Chimeric cDNA Construction

Restriction enzymes,  $T_4$  DNA polymerase and  $T_4$  DNA ligase were purchased from Pharmacia LKB BioTechnology (Piscataway, NJ). CEA (clone 17) (Benchimol et al., 1989) and mouse NCAM-120 (clone NI) (Barthels et al., 1987; Santoni et al., 1989) cDNAs were digested with specific restriction enzymes as indicated in Fig. 1 and blunt-ended with  $T_4$  DNA polymerase. The CN-1 cDNA fragment was inserted into the expression vector p91023B (R. Kaufman, Genetics Institute, Boston, MA) while the rest of the cDNA fragments were inserted into the pMV-7 retroviral expression vector (Kirschmeier et al., 1988). The chimeric cDNA constructs were confirmed by restriction mapping and, to verify retention of correct open reading frames, by sequencing the junction sites using the  $T^7$  Sequencing Kit (Pharmacia LKB Biotechnology). The  $\Delta$ NCEA cDNA construct, having the last 75 amino acids of the N domain deleted, was generously provided by Dr. S. Oikawa (Suntory Institute, Kyoto, Japan).

### Cell Culture and Transfections

LR-73 cells (Pollard and Stanners, 1979), derived from the CHO line, were grown in monolayer culture in  $\alpha$ -MEM (Stanners et al., 1971) and supplemented with 50  $\mu\text{g}/\text{ml}$  ascorbic acid and 10% FBS (growth medium). Stable CEA, NCAM, and CEA/NCAM chimera-producing transfectant LR-73 clones were obtained by calcium phosphate-mediated coprecipitation (Graham et al., 1980) of 5  $\mu\text{g}$  of recombinant plasmid DNA and 10  $\mu\text{g}$  of LR-73 carrier genomic DNA per  $3 \times 10^6$  cells. Transfectant clones were selected with either 400  $\mu\text{g}/\text{ml}$  of Geneticin (G418) for pMV-7 constructs or with the asparagine synthetase inhibitor, albizziin, in asparagine-free medium for the CN-1 construct cotransfected with 0.5  $\mu\text{g}$  of the pSV2AS vector, as previously described (Cartier et al., 1987), and tested individually for chimeric protein production by immunoblot analysis.

### Immunoblot Analysis

Cells from monolayer cultures in the late exponential phase of growth were removed with HBSS lacking  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  and containing 0.5 mM EDTA (Hank's/EDTA).  $10^6$  cells in 200  $\mu\text{l}$  buffer (PBS with 3% FBS and 0.1% sodium azide) were incubated with 1 U of glycoprophosphatidylinositol phospholipase C (GPI-PLC) (Boehringer Mannheim Biochemical) for 60 min at 37°C to release GPI-linked molecules from the cell surface. The cells were removed by centrifugation at 450 g for 5 min and the supernatant was subjected to SDS-PAGE followed by blotting to nitrocellulose membranes, which were probed with a polyclonal rabbit anti-CEA antibody, as described previously (Zhou et al., 1990).

Deglycosylation of the GPI-linked proteins was carried out using trifluoromethanesulfonic acid (TFMS), according to the method of Shively (1986).

### Cell Surface Staining

Cells were grown on chamber slides (Lab-Tek) to confluency and incubated with growth medium containing polyclonal rabbit anti-CEA antibodies for 1 h at 4°C. The monolayer was then washed with the same medium lacking the antibody and incubated for 1 h at 4°C with medium containing FITC-conjugated goat anti-rabbit IgG antibody (BioCam Scientific, Mississauga, Ontario, Canada). The degree of cell surface staining was assessed by fluorescence microscopy (Ortheplan, Zeiss).

### FACS Analysis

Monolayer cultures in the late exponential phase of growth were rendered single cell suspensions by 3 min of incubation at 37°C with 0.06% Bacto trypsin in PBS containing 15 mM sodium citrate. Control experiments indicated that this relatively light trypsinization had no effect on either the amount or size of CEA, NCAMs or chimeric proteins on the surface of the cells.  $2.5 \times 10^5$  cells were centrifuged and resuspended in 500  $\mu\text{l}$  of PBS containing 2% FBS. Polyclonal anti-CEA antibody was added to 430  $\mu\text{g}/\text{ml}$ . The cell suspension in a 5-ml Falcon tube (Becton Dickinson Labware, distributed by Fisher Scientific, Montreal, Canada) was gently rotated on a Labquake Shaker (Labindustries, Inc., Berkeley, CA) at 4°C for 30 min, washed with PBS plus 2% FBS, and incubated in 500  $\mu\text{l}$  PBS contain-

ing 2% FBS and 5  $\mu$ l of FITC-conjugated mouse anti-rabbit or anti-goat IgG (H + L) (Jackson Laboratories, Inc.) on the Labquake at 4°C for 30 min. The cells were then washed, resuspended in 750  $\mu$ l PBS plus 2% FBS and analyzed using a fluorocytometer (FACScan, Becton Dickinson Canada Inc., Mississauga, Ontario, Canada).

### Purification of Fusion Peptides

*E. coli* XL1-Blue cells transformed with pTB210 plasmids (Bolling and Mandelki, 1985) containing bacterial CMP-KDP synthetase (CKS) sequences ligated to CEA restriction fragments encoding specific domains of CEA (Hass et al., 1991) were kindly provided by Dr. Jerry Henslee and Tim Bolling (Abbott Laboratories, Chicago, IL) and were purified as described by Hass et al. (1991). The fusion peptides were assessed for purity by SDS-PAGE and Coomassie brilliant blue staining. The amounts of the fusion products were determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA), allowing for the fact that >95% of the total proteins represented the desired fusion products. The identity of the CEA domains in the peptides was confirmed by their general reactivity with anti-CEA polyclonal antibody, and their specific reactivity with epitope-specific, anti-CEA monoclonal antibodies (Zhou et al., 1993).

### Cellular Adhesion Assays

Monolayer cultures in the late exponential phase of growth were rendered single cell suspensions as described above. Homotypic adhesion assays were performed by visual measurement in a hemocytometer of the percentage of cells remaining as single cells as a function of time. The cells were incubated at 10<sup>6</sup> cells/ml in  $\alpha$ -MEM containing 0.8% FBS and 10  $\mu$ g/ml of DNase I at 37°C with stirring at 100 rpm (Benchimol et al., 1989). For the blocking studies, adhesion assays were carried out with fusion peptides at a final concentration of 2 mg/ml.

Heterotypic aggregation experiments were carried out as described previously (Zhou et al., 1990). Briefly, 4  $\times$  10<sup>6</sup> cells from monolayer culture

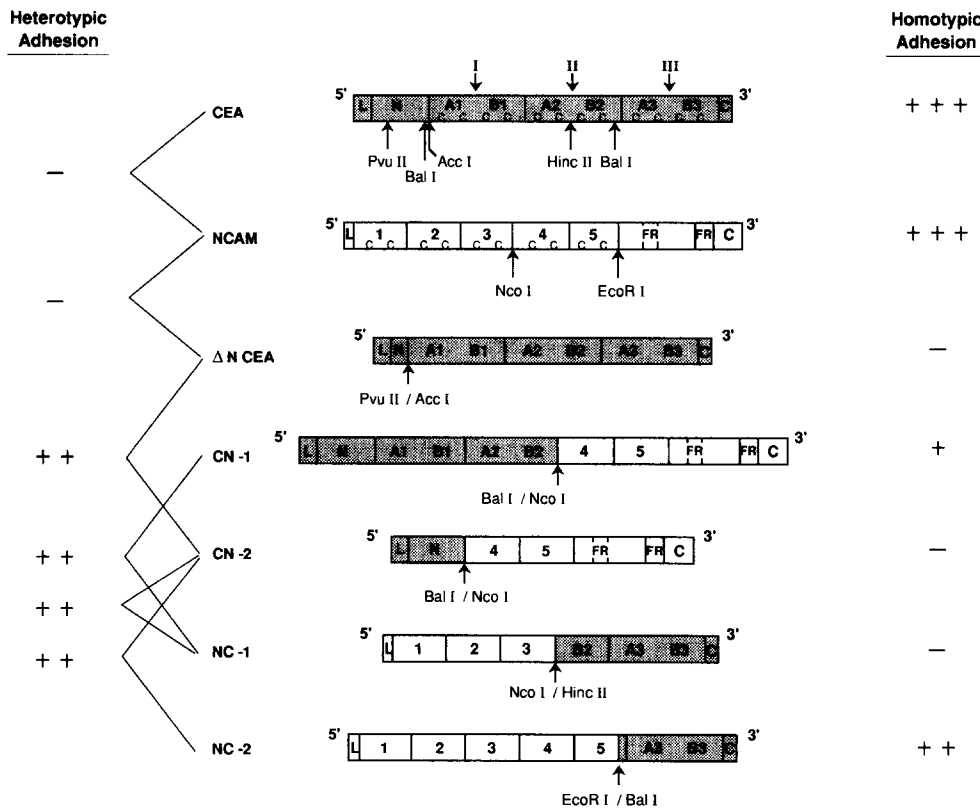
were resuspended in 100  $\mu$ l of Puck's saline containing 5% FBS and labeled by the addition of FITC to 165  $\mu$ g/ml at 37°C for 15 min. To remove excess FITC, the labeled cell suspension was layered over 13 ml of FBS and centrifuged at 1,500 rpm (250 g) for 15 min. The cell pellet was resuspended and mixed with the same number of unlabeled cells of a second type in 3 ml of  $\alpha$ -MEM plus 0.8% FBS and 10  $\mu$ g/ml of DNase I. Samples were taken 2 h after mixing, applied to microscope slides, and examined by light and fluorescence microscopy. Approximately 100 aggregates were scored for the percentage of FITC-labeled cells for each assay.

Homotypic and heterotypic adhesion assays were repeated at least three times. Results presented in Figs. 4, 5, and 7 and Tables I and II represent typical data for one of these experiments; the standard error in the values shown in Tables I and II for repeated experiments usually represented <10% of the values shown. As a crude measure of the strength of adhesion for various homotypic and heterotypic mixtures of transfectants, clump sizes after 2 h of aggregation were measured; these represent the basis for the qualitative indications shown in Fig. 1. The average clump sizes for the heterotypic aggregates varied from 4.5 to 6.5 cells, where the lower values tended to be observed if only one bond per pair of molecules was possible, while homotypic CEA aggregates with two bonds per pair of molecules showed an average clump size of 10 cells.

### Results

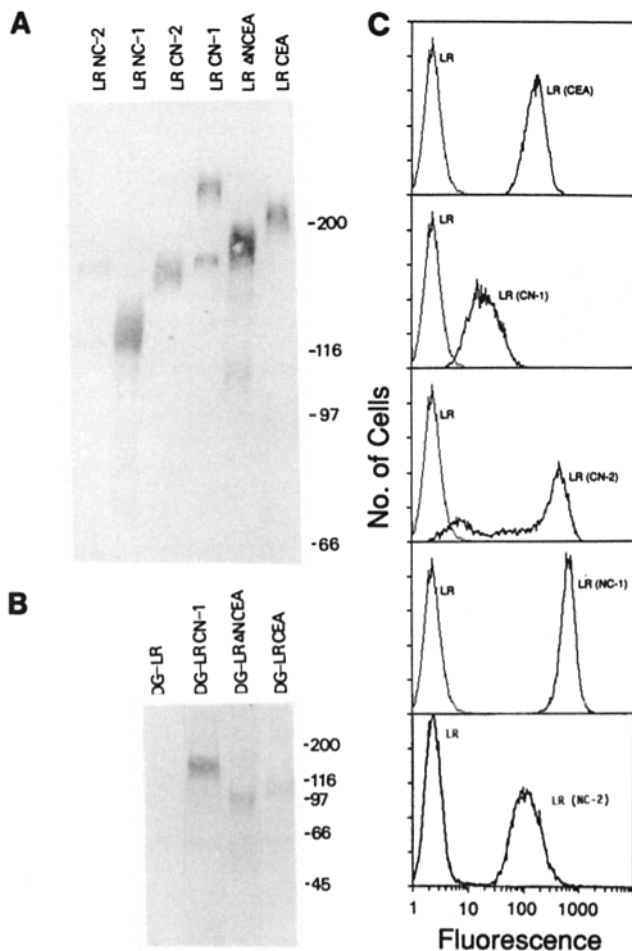
#### Characterization of CEA/NCAM Chimera-producing Transfectants

The chimeric constructs represented in Fig. 1 were inserted into expression vectors and transfected into the LR-73 cell line. Whole cells from stable transfectant clones were treated with GPI-PLC, and the soluble GPI-linked proteins released were assessed by immunoblot analysis using a rabbit anti-



**Figure 1.** Structures and adhesive properties of cDNA constructs of CEA,  $\Delta$ NCEA, NCAM, and CEA/NCAM chimeras including CN-1, CN-2, NC-1, and NC-2. Restriction enzyme sites used for constructing truncated or hybrid molecules are indicated in the parental CEA and NCAM molecules and are at the following positions (CEA): PvuII, 197; BalI, 407; AccI, 419; HincII, 1293; BalI, 1454. (NCAM) NcoI, 1118; EcoRI, 1666. The nucleotide sequences at the junctions (/) are as follows: ( $\Delta$ NCEA) CTACAG/ATACCCG; (CN-1) CAGTGG/CATGGAA; (CN-2) AACTGG/CATGGAA; (NC-1) CGGCCATG/AACCTC; (NC-2) TTGGAA-TT/CCACAG. The processed leader and COOH-terminal domains are indicated by L and C, respectively. The three very similar internal repeats in CEA, each of which consists of two C2-set domains with two cysteines (indicated

by C's) forming disulphide bridges, are indicated as I, II, and III. The five C2-set domains in NCAM-120 are numbered and the partial fibronectin-like repeats in the second last domain are indicated as FR. Results of homotypic and heterotypic adhesion assays with transfectants of these constructs are indicated.



**Figure 2.** Western blots and cytofluorimetric profiles of transfectants expressing CEA and CEA/NCAM hybrid proteins. Western blots in *A* and *B* were of phosphatidylinositol phospholipase C-released proteins from whole cells probed with rabbit polyclonal anti-CEA antisera. (*A*) Cell supernatants from untreated transfectants. (*B*) Cell supernatants treated with trifluoromethanesulfonic acid to remove sugar structures. (*C*) Cytofluorimetric profiles of parental LR-73 cells and various transfectants labeled with rabbit anti-CEA antibodies or goat anti-CEA antibodies (NC-2 transfectants) followed by anti-rabbit or anti-goat IgG FITC conjugate.

CEA polyclonal antibody as probe. The parental LR-73 cells did not produce a detectable level of CEA (Fig. 2 *B*). As shown in Fig. 2 *A*, CEA-producing transfectants yielded a band at MW 209 kD; transfectants producing  $\Delta$ NCEA, CN-1, CN-2, NC-1, and NC-2 displayed major bands at MW 178, 234 + 166, 157, 124, and 162 kD, respectively, as expected from the predicted protein molecular weights, taking into account variable glycosylation. To clarify the significance of multiple bands seen for CN-1 and  $\Delta$ NCEA transfectants (Fig. 2 *A*), GPI-treated cell supernatants were deglycosylated with TFMS. The smear of bands observed for the  $\Delta$ NCEA protein and the two forms of CN-1 were reduced to single bands after deglycosylation (Fig. 2 *B*). Thus differential glycosylation accounts for variations in molecular weight and presumably for the somewhat diffuse nature of the bands, as observed previously (Zhou et al., 1990). These results show that CEA/NCAM hybrid proteins are expressed by the

transfectants and maintain their cell surface attachment through a GPI anchor.

### Cellular Distribution of Chimeric Proteins

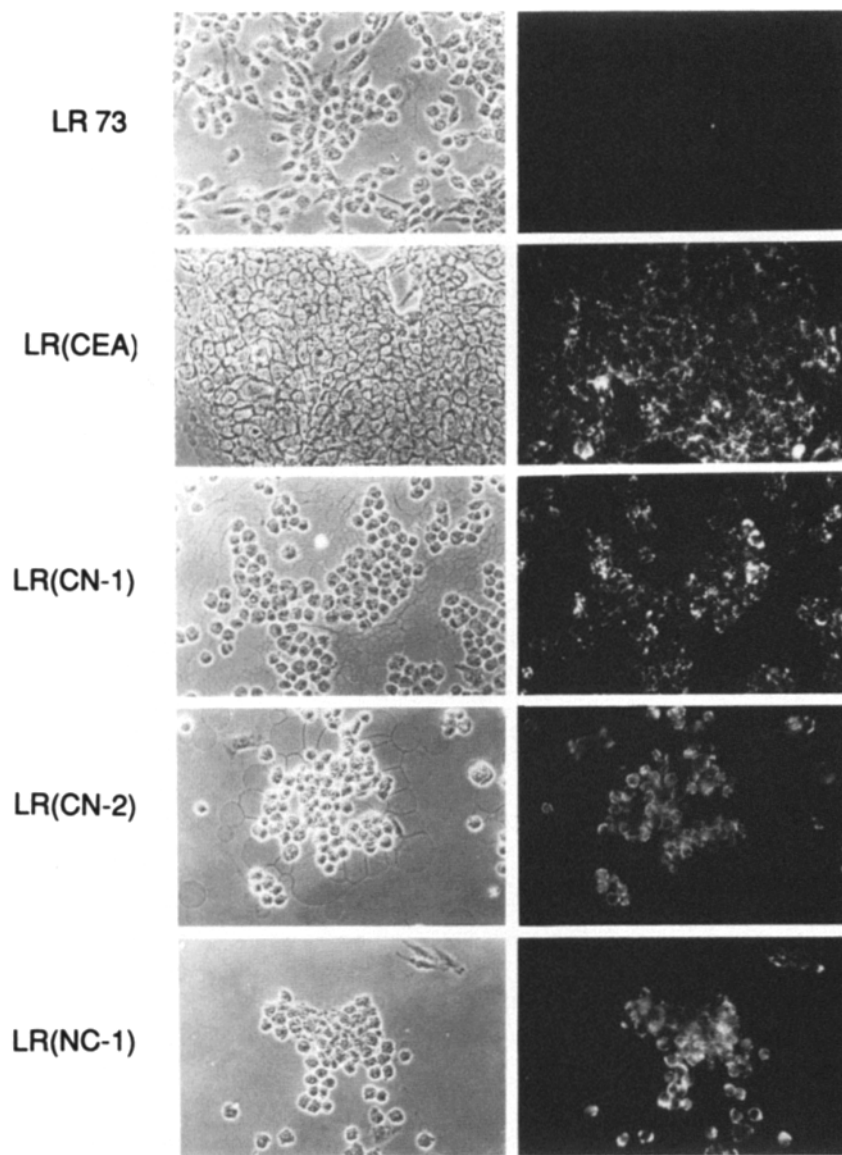
To determine the cellular distribution of the chimeric proteins, transfectant cells grown on slides were incubated with polyclonal rabbit anti-CEA antibodies and stained with FITC-labeled second antibody. As shown in Fig. 3, the parental LR-73 cells showed no fluorescent labeling. CEA and CN-1 (containing most of the CEA structure) transfectant cells exhibited a punctate cell surface labeling, whereas CN-2 and NC-1-producing cells showed a more uniform cell surface fluorescence. NC-2 transfectant cells were also labeled, but with relatively weak cell surface fluorescence, due probably to the reduced antigenic epitopes recognized by the rabbit antibodies on the residual CEA sequence in the NC-2 chimeric protein (data not shown).

To assess both the level of the chimeric proteins on the cell surface, and cell-to-cell variability in transfectant cell populations, fluorocytometric analysis was carried out. Cells in suspension were stained with polyclonal rabbit anti-CEA antibodies or goat anti-CEA antibodies for NC-2-producing cells. The parental LR-73 cells gave a negative background fluorescent distribution; CEA, CN-1, CN-2, and NC-1 transfectant clones exhibited highly positive, monodisperse fluorescence distributions in most cases (Fig. 2 *C*). NC-2 transfectants also displayed highly positive surface staining with the goat anti-CEA antibodies (Fig. 2 *C*), although these cells showed very low labeling with the rabbit anti-CEA antibodies (data not shown). These results demonstrate that the hybrid constructs were expressed on the surface of the transfectant cells.

### Homotypic Adhesion Mediated by Chimeric Proteins

To examine the binding properties of the chimeric molecules expressed on LR-73 cells, cell aggregation assays in suspension were carried out. Adhesion was measured by the decrease over time in the percentage of single cells due to the formation of aggregates.  $\Delta$ NCEA, CN-2 and NC-1 transfectants, all producing relatively high cell surface levels of their respective products (Fig. 2, *A* and *C*), did not aggregate in suspension, exhibiting the normal low background level of adhesion experienced with the parental cell line (Fig. 4). CEA, NCAM, and NC-2 transfectant cells, however, were strongly adhesive, with relatively few single cells remaining in suspension at the end of the incubation period. It is of interest that the CHO-derived parental cell, LR-73, appears to potentiate NCAM adhesion since our NCAM LR-73 transfectants appear to be far more adhesive than NCAM transfectants of other cells (Goridis, C., personal communication). CN-1 transfectant cells, producing relatively low cell surface levels of chimeric protein (Fig. 2 *C*), were also adhesive, reaching 50% single cells at the end of the assay period (Fig. 4).

These results (see Fig. 1 for summary) indicate that the NH<sub>2</sub>-terminal domain of CEA is necessary for adhesion, since neither  $\Delta$ NCEA nor NC-1 could effect cellular aggregation; a C2-set Ig domain is also required for binding, however, since CN-2 transfectants were also unable to aggregate under the same conditions. Oikawa et al. (1991) also found



**Figure 3.** Cellular distribution of CEA and CEA/NCAM chimeric proteins. Monolayer cultures were labeled with rabbit anti-CEA antisera followed by anti-rabbit IgG FITC conjugate. Fluorescence and phase-contrast micrographs of the same fields were taken for LR-73 parental cells and the indicated transfectants. The "ghost cell" outlines in the phase-contrast micrographs are artifacts of the procedure. Magnification  $\times 128$ .

that the  $\text{NH}_2$ -terminal domain of CEA was necessary but not sufficient for CEA-mediated adhesion.

#### ***Heterotypic Adhesion Mediated by Chimeric Proteins***

The domain requirements for CEA-mediated adhesion were studied further by mixing pairs of transfectants (one labeled with FITC) and observing the types of aggregates which formed. A summary of the most pertinent results is shown in Fig. 1. When labeled CEA transfectant cells were mixed with parental LR-73 cells, only completely labeled aggregates were obtained (Table I); thus, CEA did not interact with other molecules on the surface of the parental cells, as expected for a homotypic intercellular interaction mediated by homophilic binding between CEA molecules. The remaining data in Table I summarizes the results of heterotypic mixing experiments between CEA and other transfectants. FITC-labeled CEA transfectant cells sorted out from NCAM transfectant cells, giving homotypic aggregates with a background of 15% heterotypic aggregates, (Fig. 5 and Table I), while FITC-labeled NCAM transfectants did not aggregate

with  $\Delta$ NCEA transfectants, again giving a background of 15% heterotypic aggregates (Table II); these results confirm the existence of different adhesive determinants for CEA and NCAM (Zhou et al., 1990) and preclude interactions between CEA and NCAM domains which could confound the interpretation of the heterotypic assays with the constructs. Labeled CEA-producing cells formed mixed aggregates, representing 60–70% of the total aggregates, with cells producing  $\Delta$ NCEA, CN-1, CN-2, NC-1, or NC-2 (Fig. 5 and Table I). The presence of a minority of homotypically labeled aggregates was presumably due to the fact that, in the presence of other cells bearing full length CEA molecules, weaker interactions between CEA and CEA/NCAM transfectants are more difficult to observe, resulting in a skewing of the distributions toward labeled aggregates, especially when one of the partners is unable to aggregate on its own (Fig. 5 and Table I). CEA transfectants were thus able to interact with transfectants which were both competent (CN-1 and NC-2) and incompetent ( $\Delta$ NCEA, CN-2, and NC-1) in self-aggregation. The observed heterotypic interactions be-

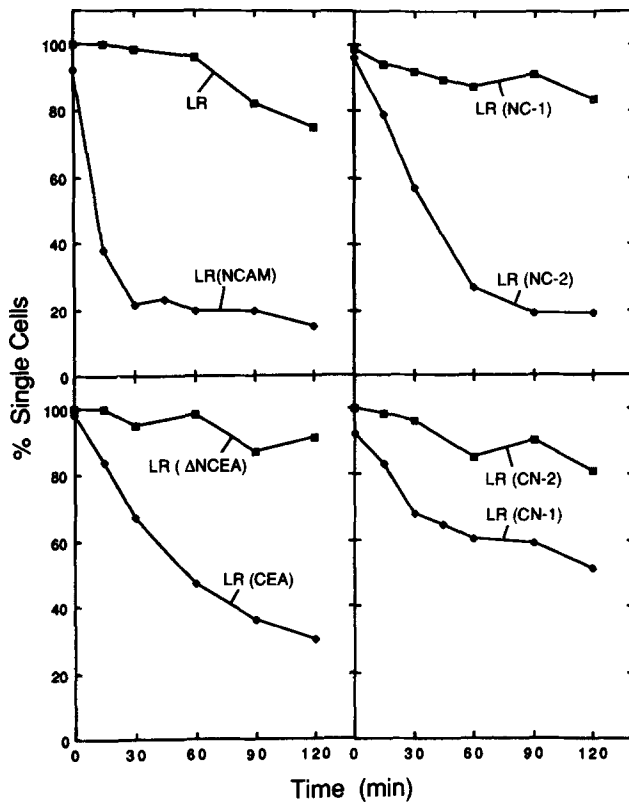


Figure 4. Kinetics of aggregation for LR-73 parental cells and the indicated transfectants by measurement of the percentage of single cells remaining in suspension as a function of time.

tween CEA and CN-2 (Table I) or  $\Delta$ NCEA (Fig. 5) transfectant cells, indicate that both the NH<sub>2</sub>-terminal domain and the internal repeat domains of CEA, respectively, can bind to the complete CEA molecule.

The results obtained with non-self-adhesive CN-2 transfectant cells mixed with non-self-adhesive  $\Delta$ NCEA transfectant cells are particularly informative (see Fig. 1 for highlights). Cellular aggregates were formed and these were essentially all heterogeneous (Fig. 5); CN-2 also interacted

Table I. Specificity of Adhesion of CEA,  $\Delta$ NCEA, and Various CEA/NCAM Chimeras Expressed on the Surface of CHO Cells

Cell populations		Aggregates visualized		
Unlabeled	FITC labeled	Homotypic unlabeled	Heterotypic mixed	Homotypic labeled
%				
LR	LR CEA	2	0	98
LR NCAM	LR CEA	44	15	41
LR $\Delta$ NCEA	LR CEA	0	68	32
LR CN-1	LR CEA	16	69	15
LR CN-2	LR CEA	0	57	43
LR NC-1	LR CEA	1	63	36
LR NC-2	LR CEA	14	67	19

Summary of heterotypic experiments between two populations of cells, one labeled with FITC and the other unlabeled. Large percentages (>50%) of mixed aggregates indicate heterotypic adhesion whereas low percentages suggest cell sorting.

heterotypically with NC-1 and NC-2 (Fig. 5 and Table II). These results suggest that the N domain of CEA (the only CEA domain present in CN-2) binds to an internal repeat CEA domain in  $\Delta$ NCEA, NC-1, and NC-2. Since NC-2 has only the A3B3 domain from CEA, this binding must occur between the N domain and the A3B3 domain, although interactions with the A1B1 and A2B2 internal domains are not excluded.

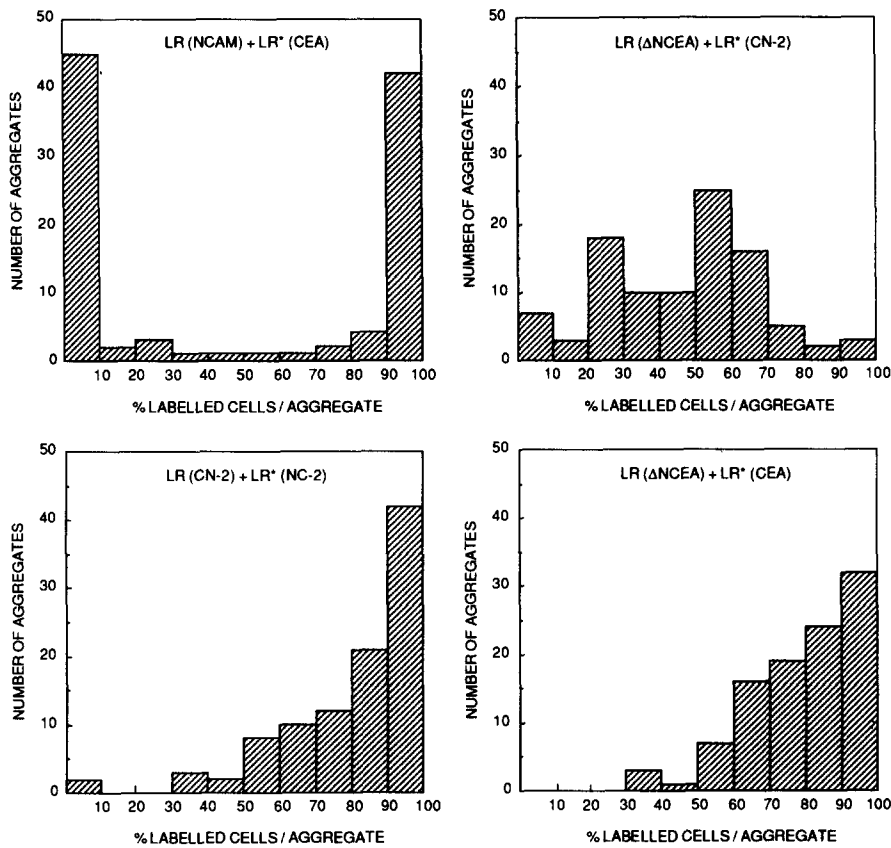
#### Inhibition of CEA-mediated Adhesion by CEA Domain-Specific Peptides

CEA fusion peptides containing *E. coli* CKS were purified from bacteria and analyzed by SDS-PAGE electrophoresis as shown in Fig. 6. The purified proteins appeared to be >95% homogeneous and their molecular weights were ~41 kD for CKS-N, 50 kD for CKS-A1B1, and 47 kD for both CKS-A2B2 and CKS-A3B3, as expected for peptides consisting of the 24-kD CKS peptide fused to CEA domain peptides calculated to have molecular weights of 12.4, 20.5, and 20.5 kD, respectively. The relatively small differences between the observed and the calculated molecular weights are presumably due to aberrant electrophoretic migration. The fusion peptides were then tested for their effects on CEA-mediated adhesion. Both the N domain and A3B3 domain CKS fusion peptides inhibited the aggregation of CEA transfectant cells completely, leaving 80–90% single cells (Fig. 7). The A1B1 domain and A2B2 domain peptides, on the other hand, had no effect on aggregation until 60 min, when they caused disaggregation to a limited extent. These results thus confirm the results of aggregation assays presented above: both the N and the A3B3 domains are directly involved in CEA-mediated aggregation. The weaker and delayed blocking by the A1B1 and A2B2 domains on adhesion could be due to their relatively close structural similarity with the A3B3 domain (Beauchemin et al., 1987).

#### Discussion

The different homotypic intercellular adhesion specificities of CEA and NCAM (Zhou et al., 1990), both Ca<sup>++</sup>-independent adhesion molecules of the Ig superfamily, have been exploited in this work to determine the domains and the mechanism involved in intermolecular binding for CEA, and to investigate the domain requirements for NCAM binding. Truncated and chimeric cDNAs were constructed and were stably expressed on the surfaces of transfected cells, as shown by indirect immunofluorescent antibody surface staining of whole cells and by release of the expressed chimeric proteins from whole cells by treatment with phosphatidylinositol phospholipase C.

The homotypic binding site for chick NCAM-140 has recently been mapped to the third C2-set domain (Rao et al., 1992). In our studies, whereas both NCAM and NC-2 transfectants (with all five NCAM C2-set domains) mediated adhesion, NC-1 transfectants (having the first three NCAM C2-set domains only) did not (see Fig. 1). Therefore it would appear that, for GPI-linked mouse NCAM-120, the loss of the last two C2-like domains leads to loss of the adhesion function, and that all five C-like domains are required. If the homophilic binding site is also localized on the third C-like domain for mouse NCAM-120, it seems that this binding domain is not sufficient for adhesion function, at least in this



**Figure 5.** Heterotypic aggregation of mixed populations of transfectant cells. FITC-labeled CEA transfectants were mixed with NCAM or  $\Delta$ NCEA transfectants; FITC-labeled NC-2 transfectants with CN-2 transfectants; and FITC-labeled CN-2 transfectants with  $\Delta$ NCEA transfectants. After 2 h in suspension, the quantitative distribution of labeled cells in cellular aggregates is shown as histograms relating the number of aggregates to the percentage of labeled cells per aggregate.

chimeric construct. This discrepancy with the results of Rao et al. (1992) could be explained by an adhesion-unfavorable conformation in this particular construct (although the very similar construct, NC-2, is adhesion-competent), or the lack of the cytoplasmic domain, or by species differences. In addition, the self-adhesive property of the NC-2 construct indicates that the truncated fibronectin-like domains in NCAM are not important for NCAM-NCAM homophilic binding.

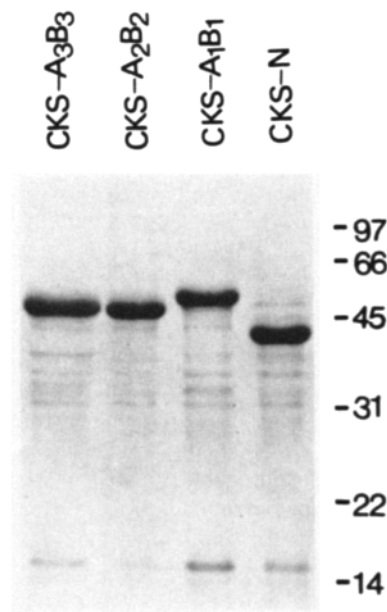
Cells from the transfectant clones producing truncated CEA or chimeric CEA/NCAM molecules were also tested for their ability to aggregate in homotypic adhesion assays.

**Table II. Specificity of Adhesion of NCAM,  $\Delta$ NCEA, and Various CEA/NCAM Chimeras Expressed on the Surface of CHO Cells**

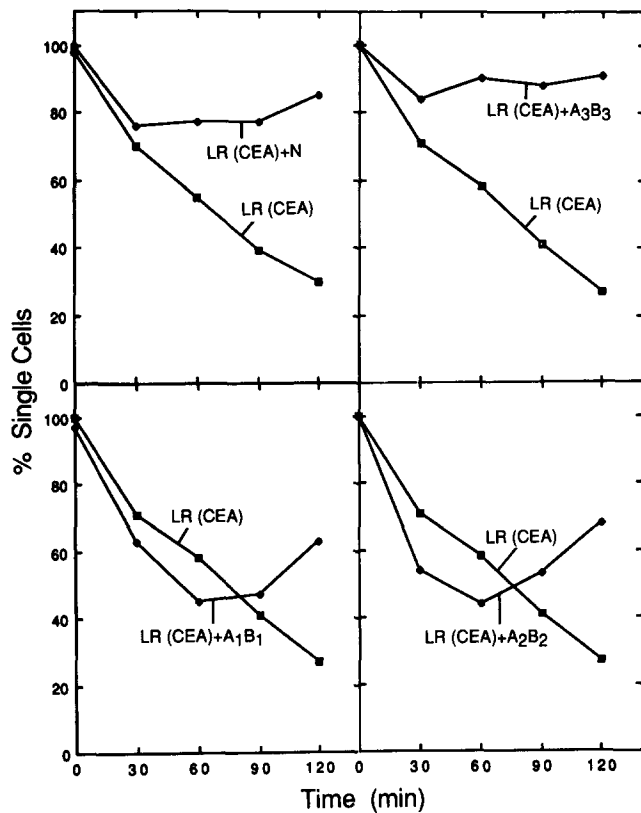
Cell populations		Aggregates visualized		
Unlabeled	FITC labeled	Homotypic unlabeled	Heterotypic mixed	Homotypic labeled
%				
LR CN-2	LR $\Delta$ NCEA	6	86	8
LR NC-1	LR CN-1	7	87	6
LR NC-1	LR CN-2	9	83	8
LR CN-2	LR NC-2	2	56	42
LR NC-2	LR NCAM	3	13	84
LR NC-1	LR NCAM	4	21	75
LR $\Delta$ NCEA	LR NCAM	2	15	83

Summary of heterotypic experiments between two populations of cells, one labeled with FITC and the other unlabeled. Large percentages (>50%) of mixed aggregates indicate heterotypic adhesion whereas low percentages suggest cell sorting.

No aggregation was observed for either  $\Delta$ NCEA-, CN-2-, or NC-1-producing cells (see Fig. 1 for summary of results in relation to structure). Thus, removal of either the whole or part of the 108-amino acid V-like NH<sub>2</sub>-terminal domain of CEA (NC-1 or  $\Delta$ NCEA, respectively), or removal of all of



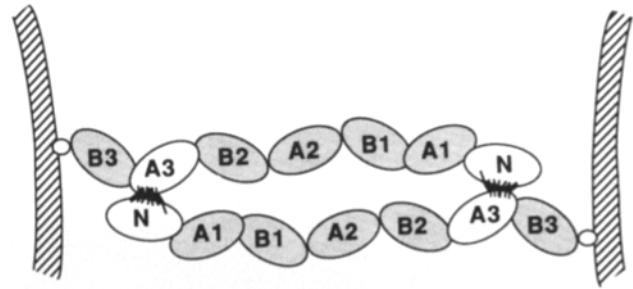
**Figure 6.** Electrophoretic analysis of cell extracts of *E. coli* cells producing CKS/CEA domain fusion products using a 12% SDS-polyacrylamide gel. After electrophoresis, the gel was stained with Coomassie blue.



**Figure 7.** Effect of CEA-domain peptides on the aggregation of CEA transfectant cells. Aggregation results are shown for CEA transfectant cell suspensions in the absence and presence of the indicated peptides.

the 178-amino acid double CEA C2-set-like internal repeat domains (CN-2) obliterates the adhesive capacity of CEA. CN-1, however, containing an intact V-like domain and the first two internal repeat domains of CEA, gave adhesion. These results demonstrate, therefore, that CEA-mediated adhesion requires both the V-like domain and at least one of the internal repeat domains.

The results of heterotypic adhesion assays among transfectants expressing different CEA/NCAM chimeric proteins (see Fig. 1) were even more definitive, since they directly confirmed the above conclusion and demonstrated that the chimeric proteins were capable of specific adhesive function when appropriately matched with other proteins. Thus CEA-producing cells could aggregate with all types of chimera-producing cells, including those lacking the V-like N domain but containing at least two intact C-like domains (NC-1 and NC-2) and those lacking all C-like domains but containing an intact V-like N domain (CN-2) (Table I). Thus either a V-like or a C-like domain alone from one hybrid molecule is sufficient to allow interactions with the intact CEA molecule. Interactions between the C2-set domains of CEA and NCAM need not be considered in the interpretations; these can be excluded by the lack of interaction between NCAM and CEA or NCAM and  $\Delta$ NCEA (Table II). Similarly, heterotypic interactions between the C domains of N-CAM, such as binding between domains C4 and C5 in CN-2 with domains C1, C2, and C3 in NC-1 can be excluded by the negligible aggregation between NCAM and CN-2 or NC-1 transfectants (Table II).



**Figure 8.** Double reciprocal binding model for two anti-parallel CEA molecules on apposed cell surfaces. The N terminal V-like and the 6 internal C-like domains are approximated as ellipsoids from the structure predicted by Bates et al. (1992) with the double N-A3 bonds depicted as striated links. The overall curvature of the molecules has been arbitrarily introduced to emphasize the position of the bonds.

Significantly, heterotypic aggregates were obtained when CN-2 transfectants (containing the V-like domain but no C-like domains from CEA) and  $\Delta$ NCEA transfectants (containing a severely truncated V-like domain and all six C-like domains), both nonadhesive on their own, were mixed (Fig. 5 and Table II). This result indicates that the V-like CEA N-domain of CN-2 can interact with a C-like domain of  $\Delta$ NCEA. These findings support a model (Fig. 8) in which antiparallel CEA molecules on apposing cell surfaces are held together by double reciprocal, symmetrical bonds between the N-domain of one molecule and a C2-set domain of the other. Such an interaction would lead to an especially strong intermolecular protein-protein binding. In direct support of this model, the addition of bacterial fusion peptides (free of carbohydrate structures) containing either the N or the A3B3 C2-set domains of CEA could completely block CEA-mediated adhesion (Fig. 7). In addition, the peptide blocking results confirm the involvement of protein-protein interaction in CEA homophilic binding.

Which C2-set domain in CEA is involved in this proposed double-reciprocal bonding? CN-2, with only the N domain of CEA, also binds to both NC-1 and NC-2 transfectant cells, which indicates that the C2-set domain involved could be A3 and B3, since NC-1 contains the last three and NC-2 contains only the last two of these C2-set domains; this agrees with the peptide blocking experiments. The fact that CN-1, lacking A3 or B3, can aggregate homotypically, however, indicates that the alternative involvement of other C-like domains is possible. Since the first third of each of the three CEA internal repeat domains, i.e., the A1, A2, and A3 C2-set subdomains (Fig. 1), are highly conserved at the amino acid sequence level (Beauchemin et al., 1987), these C2-set domains could have similar protein folding and function. The fact that the blocking studies showed weaker, delayed but nonetheless positive blocking with the A1B1 and A2B2 peptides (Fig. 7) supports the case for the alternative use of these internal domains in CEA-mediated bonding. Therefore we predict that CEA-mediated adhesion normally involves bonding between the N V-like domain and the A3 C2-like domain, with alternative, less favorable bonding between the N and the A1 or A2 domains.

It has been reported that a monoclonal antibody specific for the V-like N-domain of CEA can inhibit the adhesion of the CEA-expressing human colon adenocarcinoma cell line,



LoVo, to immobilized purified CEA, implicating the involvement of the V-like domain in direct homophilic binding of CEA molecules in intercellular adhesion (Levin et al., 1992). In addition, anti-CEA monoclonal antibodies which are specific for the N-domain of CEA have been indicated to inhibit CEA homotypic adhesion activity using CEA transfectants (Oikawa et al., 1991). Our own studies, in which a battery of monoclonal antibodies was studied for effects on CEA-mediated adhesion have implicated the A3B3 domain as well as the N domain (Zhou et al., 1993). The antibody studies thus support our model.

The three-dimensional structure of CD2, an adhesion molecule of the Ig superfamily, has been recently determined and its heterophilic binding with LFA-3, another member of the Ig superfamily, inferred (Driscoll et al., 1991). Other studies have shown that the NH<sub>2</sub>-terminal V-like domain of CD2 binds to the NH<sub>2</sub>-terminal V-like domain of LFA-3 (Recny et al., 1990). Myelin P<sub>0</sub> protein, another Ig superfamily member, also mediates homophilic adhesion through its V-like domain only (Filbin et al., 1990). Most recently, the crystal structure of a soluble form of CD2 (sCD2) has been reported indicating homophilic adhesion of two CD2 crystal molecules through their V domains (Jones et al., 1992). Ig V-like domain dimerization has been proposed to occur through the GFCC'C'  $\beta$  sheets of the uppermost pair of  $\beta$  sheet folds (Springer, 1991). The interaction between CD2 and LFA-3, for example, has been suggested to occur on their CFG faces (Driscoll et al., 1991). In sCD2, the  $\beta$ -bulge conformations in the C' and G  $\beta$ -strands and the CC' and FG loops, and the linker region between the two V-like domains are crucial for its proper dimerization (Jones et al., 1992). The three-dimensional structure for CEA has been predicted recently by comparison with the first V-like domain of rat CD2 (Bates et al., 1992). Both CFG faces of the N and A3 domains are devoid of potential glycosylation sites and are positioned favorably for interaction; it would therefore be of interest to test for the involvement of these subregions of the domains identified in this work in CEA-CEA homophilic binding.

Our studies demonstrate, for the first time, an interaction between a V-like domain and a C-like domain in homophilic binding of an Ig superfamily member. From an evolutionary point of view, V-V and V-C interactions can be explained by a single primordial Ig domain duplicating and diverging into V-like and C-like domains. For most molecules, the binding specificities were retained on either V-like or C-like domains; whereas for CEA, both domains became involved in a more stabilized domain-heterophilic intermolecular interaction.

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