

The Specificity for the Differentiation Blocking Activity of Carcinoembryonic Antigen Resides in its Glycophosphatidyl-inositol Anchor

Robert A. Screaton,* Luisa DeMarte,* Petr Dráber,† and Clifford P. Stanners*

*McGill Cancer Centre and Department of Biochemistry, McGill University, Montreal, Quebec, Canada H3G 1Y6; and

†Institute of Molecular Genetics, 142 20 Prague 4, Czech Republic

Abstract. Ectopic expression of various members of the human carcinoembryonic antigen (CEA) family of intercellular adhesion molecules in murine myoblasts either blocks (CEA, CEACAM6) or allows (CEACAM1) myogenic differentiation. These surface glycoproteins form a subset of the immunoglobulin (Ig) superfamily and are very closely related, but differ in the precise sequence of their external domains and in their mode of anchorage to the cell membrane. CEA and CEACAM6 are glycophosphatidyl-inositol (GPI) anchored, whereas CEACAM1 is transmembrane (TM) anchored. Overexpression of GPI-linked neural cell adhesion molecule (NCAM) p125, also an adhesion molecule of the Ig superfamily, accelerates myogenic differentiation. The molecular requirements for the myogenic differentiation block were investigated using chimeric constructs in which the COOH-terminal hydrophobic domains of CEA, CEACAM1, and NCAM p125 were exchanged. The presence of the GPI signal

sequence specifically from CEA in the chimeras was sufficient to convert both CEACAM1 and NCAM into differentiation-blocking proteins. Conversely, CEA could be converted into a neutral protein by exchanging its GPI anchor for the TM anchor of CEACAM1. Since the external domains of CEA, CEACAM1, and NCAM can all undergo homophilic interactions, and mutations in the self-adhesive domains of CEA abrogate its differentiation-blocking activity, the structural requirements for differentiation-inhibition are any self-adhesive domains attached to the specific GPI anchor derived from CEA. We therefore suggest that biologically significant functional information resides in the processed extreme COOH terminus of CEA and in the GPI anchor that it determines.

Key words: CEA • GPI anchors • inhibition of differentiation • Ig superfamily • NCAM

Introduction

Carcinoembryonic antigen (CEA)¹ is overexpressed in many human cancers, including those of colorectal, breast, and lung origins, and for 30 yr has been one of the most prevalent tumor markers (Averbach and Sugarbaker, 1995; Boucher et al., 1989; Ilantzis et al., 1997). CEA is the prototypic member of a large gene subfamily (Öbrink, 1997) of the Ig superfamily that includes CEACAM1 (formerly BGP) and CEACAM6 (formerly NCA).

Address correspondence to Clifford P. Stanners, McGill Cancer Centre, 3655 Promenade Sir-William-Osler, Montreal, Quebec, Canada H3G 1Y6. Tel.: (514) 398-3535. Fax: (514) 398-6769. E-mail: stanners@med.mcgill.ca

¹Abbreviations used in this paper: C, CEA; C1, CEACAM1; CEA, carcinoembryonic antigen; DIGs, detergent-insoluble glycolipid domains; GPI, glycophosphatidyl-inositol; NCAM, neural cell adhesion molecule; nt, nucleotides; TM, transmembrane; PI-PLC, phosphatidylinositol phospholipase C.

CEACAM6, like CEA, is upregulated in human malignancy (Boucher et al., 1989; Hinoda et al., 1991; Ilantzis et al., 1997; Kim et al., 1992), whereas CEACAM1 is usually downregulated, suggesting that CEACAM1 could act as a tumor suppressor, as has been shown for the mouse and rat homologues of human CEACAM1 (Hsieh et al., 1995; Kleinerman et al., 1995; Kunath et al., 1995; Neumaier et al., 1993). CEA, CEACAM6, and CEACAM1 all possess homotypic intercellular adhesion activity in vitro (Benchimol et al., 1989; Oikawa et al., 1989; Rojas et al., 1990; Zhou et al., 1990). CEA and CEACAM6 are glycophosphatidyl-inositol (GPI)-linked to the cell surface (Hefta et al., 1988, 1990; Takami et al., 1988), whereas CEACAM1 has transmembrane (TM) and cytoplasmic domains, features that correlate with observed differences in function to date (Rojas et al., 1996).

Myogenic fusion requires the functional integrity of

cell–cell adhesion systems, such as the cadherins (N-cadherin, M-cadherin) and Ig superfamily adhesion molecules (NCAM, vascular [V]CAM), and cell–ECM-binding interactions mediated by the integrin family, which includes VLA-4/ $\alpha_4\beta_1$ and $\alpha_5\beta_1$ (Knudsen, 1990). Overexpression of various splice variants of human NCAM accelerates myogenesis in mouse C2 myoblasts (Peck and Walsh, 1993; Dickson et al., 1990) and in transgenic mice (Fazeli et al., 1996). These include the p125 isoform, which possesses five Ig C2-like repeats, two partial non-RGD fibronectin type III-like domains, a 5-kD skeletal muscle-specific domain (Dickson et al., 1987), and a GPI anchor (Barton et al., 1988). We have demonstrated that ectopic expression of CEA and CEACAM6 disrupts the differentiation program of rat L6 myoblasts, whereas CEACAM1 and an adhesion-defective deletion mutant of CEA (Δ NCEA) were unable to inhibit differentiation (Eidelman et al., 1993; Rojas et al., 1996). These results are consistent with the observed upregulation of CEA and CEACAM6 and downregulation of CEACAM1 in human tumors. Moreover, CEA significantly reduces the latency required to form tumors in nude mice, and cooperates with Myc and Bcl-2 in transformation; we have proposed that CEA's differentiation-blocking activity represents a novel contribution to oncogenesis (Screaton et al., 1997).

GPI proteins are specifically targeted to apical membranes and excluded from the basolateral membranes of polarized epithelial cells (Rodriguez-Boulan and Nelson, 1989). Conversely, proteins with a bona fide TM domain are generally transported to basolateral surfaces, by virtue of signals in their cytoplasmic domains. The lateral compartmentalization of GPI proteins within the lipid bilayer is initiated during membrane biosynthesis, and results not from protein–protein interactions, but from the preferential miscibility of the glycolipid moiety of the GPI anchor with sphingolipids and cholesterol, perhaps together with the lectin VIP36 (Fiedler and Simons, 1996; Simons and Ikonen, 1997). These three components coalesce in the trans-Golgi network to form membrane islands, or rafts, which serve as targeting shuttles to the apical surface (Simons and Ikonen, 1997). Rafts can be concentrated *in vitro* at 4°C as a precipitate of nonionic, detergent-insoluble glycolipid domains, or DIGs, a low-density membrane fraction rich in unsaturated glycolipids and GPI proteins (Brown and London, 1997; Brown and Rose, 1992; Fiedler et al., 1993). Interestingly, rafts are also involved in targeting of apical markers in nonpolarized cells (Musch et al., 1996; Yoshimori et al., 1996); however, in the absence of epithelial tight junctions, raft components diffuse to homogeneity once at the cell surface. Apart from GPI proteins, DIGs concentrate doubly acylated tyrosine kinases of the src family (Dráberova and Dráber, 1993; Rodgers et al., 1994; Shenoy-Scaria et al., 1992, 1993, 1994), which are tethered to the inner cytoplasmic leaflet of the membrane bilayer by covalent myristoyl and palmitoyl group modifications, and G α monomers (Solomon et al., 1996). Thus the interaction of GPI proteins with the lipid constituents of rafts at the plasma membrane govern their localization and possibly their functions at the cell surface. Recently, the existence of rafts under physiological conditions in fibroblasts and epithelial cells has been demonstrated using fluorescence resonance energy transfer and

chemical cross-linking techniques, respectively (Friederichson and Kurzchalia, 1998; Varma and Mayor, 1998).

We have addressed the role of the external domains and the GPI anchor in the CEA-mediated myogenic differentiation block using chimeras between CEA, CEACAM1, and GPI-linked NCAM p125. We confirm that overexpression of NCAM accelerates myogenesis (Peck and Walsh, 1993), and report that the CEA, CEACAM1, or NCAM extracellular domains are sufficient to inhibit differentiation when attached specifically to a CEA GPI anchor. Conversely, replacing the GPI linkage of CEA with the TM domain of CEACAM1 cancels the differentiation block. These results lead to the following hypothesis: the differentiation inhibition activity of CEA requires the combination of CEA GPI linkage with any functional external self-adhesion domain. Since the COOH-terminal domain of CEA and not that of NCAM is necessary for the block, the GPI anchors that replace these domains harbor specific biological information.

Materials and Methods

Cell Culture and Differentiation Assay

CHO-derived LR-73 fibroblasts (Pollard and Stanners, 1979) were grown in monolayer in α -MEM plus 10% FBS in a humidified atmosphere of 5% CO₂ at 37°C. Rat L6 (Yaffe, 1968) myoblasts were grown as monolayer cultures in DME containing 10% FBS (growth medium) as previously described (Screaton et al., 1997). All myoblast cultures were passaged while subconfluent to avoid selection of nonfusing variants. The differentiation/fusion assay was performed in DME plus 2% horse serum (differentiation medium) as previously described (Screaton et al., 1997); cultures were assessed for fusion after 4 d in differentiation medium.

Constructs

The CEACAM1–CEA (C1–C) chimera (Dráber, P., and C.P. Stanners, unpublished data) possesses the leader, N, A1B1, and A2 domains of CEACAM1 and six extracellular residues plus the hydrophobic COOH-terminal domain of CEA. C1–C was produced by ligation of nucleotides (nt) 1–1314 of CEACAM1-4L (the longest splice variant of CEACAM1, denoted C1-4L in Fig. 9 and with the basic structure shown therein) to nt 2106–3037 of CEA at HindIII sites introduced into both cDNAs at the specified positions. The C–C1S chimera consists of the external domains of CEA linked to the TM and short cytoplasmic domains of the CEACAM1 splice variant, CEACAM1-1S; this chimera was generated by PCR overlap extension modeled after Sippel et al. (1996), as follows: a first round of PCR was performed using: (i) the CEA cDNA and 5' outside primer 5' TAGTGGATCCTATACGTGCCAA 3' (nt 975–996, containing the underlined natural BamHI site) and internal antisense primer 5' TGAGAGGCCATTTTCGACTGTGATGCTCTTACTA 3' (corresponds to nt 992–1006 of CEACAM1-1S at the 5' end and nt 2099–2018 of CEA at the 3' end) to produce a 1.1-kb product, and (ii) the CEACAM1-1S cDNA and 3' outside primer 5' ATGAATTCATAAATTATTCTGTGGC 3' (nt 1218–1235, with the underlined added EcoRI site) and 5' sense internal primer 5' AAGAGCATCACAGTCCGAAAATGGCCTCTCA 3' (antisense of the internal primer used in i, minus 5 nt at 5' end) to produce a 0.3-kb product. A second round of PCR using the 1.1- and 0.3-kb round 1 products and the two outside primers listed above produced a 1.4-kb product, which was digested with BamHI (5' end) and EcoRI (3' end) and ligated to the 5' end of the BamHI-digested CEA cDNA to produce C–C1S.

N–C and C–N were generated using overlap extension as follows: as the ω -site in NCAM is unknown, we assigned it to be the last amino acid (A736) encoded by exon 14; thus the TM domain in NCAM begins at Thr737. Exon 14 is spliced onto the GPI-specific exon 16 in the p125 splice variant (Barton et al., 1988). Sequence alignment supports this as a possible ω -site (Udenfriend and Kodukula, 1995; Moran et al., 1991; Nuoffer et al., 1993). Other possible sites (Gly739, Gly740, Asn741, and Ser742) were excluded on the basis of nonconservation between chickens and humans: in

chicken NCAM, Gly740, Asn741, and Ser742 are Ser740, Pro741, and Ser742 (see Fig. 1). The presence of a proline residue at position 742 in chicken makes GPI substitution at these positions unlikely, if not impossible. The junction site between NCAM and CEA was selected to be six residues from the beginning of the TM domain, as for C1-C. For N-C, PCR was performed with the 5' primer 5' GCAGTGACCACGTCATGC 3' (nt 2214-2231 of NCAM, containing a natural AspI site, underlined), and 3' primer 5' TGAAGCTTGGCTGGCCGAGG 3' (nt 2327-2339 NCAM 125), with an added HindIII site (underlined). After digestion with AspI and HindIII, this 0.13-kb fragment was ligated together with 2.2-kb Sall-AspI NCAM and 0.9-kb HindIII-EcoRI CEA fragments in one reaction into pBluescript (Stratagene). For N-C blunt, a first round of two PCR reactions was performed using CEA or NCAM template cDNA. For the NCAM template, the 5' outside primer was 5' GCAGTGACCACGTCATGC 3' (nt 2214-2231 of NCAM containing a natural AspI site, underlined), and for CEA the 3' antisense outside primer was 5' CTAGAACTAGTGGATCCC 3' (sequence from pBluescript MCS containing a BamHI site, underlined). The inside primers were 5' AGG-AGAAGTTCAGATGCTGGGATGGCTGTGGG 3' and 5' ACAG-CCATCCCAGCATCTGGAACCTCTCCTGGTC 3' (nt 2191-2223 of NCAM and nt 2014-2047 of CEA in both the antisense and sense directions, respectively). The second round of PCR used the two first round products, as described above for C-C1S. The final PCR product was digested with AspI and BamHI, and was cloned into pBluescript (N-C) at the corresponding AspI and BamHI sites, thus removing the fragment containing the HindIII site junction. All constructs were subcloned into the p91023B expression vector (courtesy of R. Kaufman, Genetics Institute, Boston, MA) for transfection.

Transfections

L6 myoblasts and LR-73 fibroblasts were seeded at 1.8×10^5 , or 4 and 6×10^5 cells/100-mm dish, respectively, and cotransfected 24 h later by calcium phosphate coprecipitation with 5 μ g of p91023B expression vector containing appropriate cDNA chimeras and 0.5 μ g pSV2neo or pBabe(neo) plasmid per dish. For human NCAM-125 (a kind gift of F.S. Walsh, ICRF, London, UK), 5 μ g p4.4.4(NCAM)-neo expression vector (Gunning et al., 1987) was used. Neomycin(G418)-resistant pooled transfectant populations were selected with 400–600 μ g/ml G418 for 12–14 d. G418-resistant clones were isolated either individually, or pooled and sorted for high surface expression levels by FACS using monoclonal anti-CEA antibody (J22; Zhou et al., 1993) or monoclonal anti-human NCAM antibody (ERIC-1; BIOCAN Scientific). Double transfectant total populations were generated either by cotransfection of NCAM 125 cDNA with pBabe(hygro) into L6(CEA) cells, followed by selection with 100 μ g/ml hygromycin-B, or by cotransfection of both cDNAs together with pBabe(neo) into parental L6 myoblasts, followed by selection with G418 as described above. Two independent transfections were done for C1-C, N-C blunt, and CEA, with identical results in assays for biological effects. All other transfections were carried out once. To avoid difficulties in interpretation due to clonal differences in cellular properties unrelated to those caused by the transfected construct, populations of pooled transfectant clones were used in all cases, except clones L6(CEACAM1-4L), denoted L6(C1-4L) here; LR(CEACAM1-4L), denoted LR(C1-4L) here; L6(CEACAM6); and LR(CEA-7), which were all used as positive controls here and have been shown previously to be representative in cellular properties (Rojas et al., 1990, 1996); for L6(C-C1S), two independent clones were tested, which gave equivalent results. The LR-73 pooled transfectant populations were comprised of hundreds of clones, whereas the L6 pooled transfectant populations consisted of 20–50 clones; the polyclonal nature of the latter was confirmed by Southern analysis indicating multiple integration sites, as described elsewhere for the L6(CEA) pooled transfectant population (Screaton et al., 1997). All functional assays were performed in the absence of G418 selection, and no loss of surface expression was detected over the culture period required for the assays (data not shown).

FACS Analysis

Exponentially growing cultures were trypsinized (CEA family transfectants) or incubated with PBS-citrate plus 4 mM EDTA (PBS-C-E) (NCAM p125 transfectants) for 3–4 min at 37°C. NCAM is sensitive to trypsin, which removes a major 50-kD NCAM fragment (estimated from SDS-PAGE mobility), reducing overall NCAM surface levels two to threefold (data not shown). 1.25×10^5 cells were resuspended in 0.25 ml

ice cold PBS plus 2% FBS (PBSF) containing mAbs J22 or ERIC-1 at a dilution of 1:50 to 1:100 for 30 min on ice. Cells were centrifuged, rinsed with 2 ml PBSF, and resuspended in 0.25 ml PBSF containing goat anti-mouse FITC- or phycoerythrin-conjugated antibody (BIOCAN Scientific) diluted 1:100. After 30 min of incubation, cells were rinsed and resuspended in 0.3 ml PBSF for cytofluorometric analysis using a FACScan™ instrument (Becton Dickinson).

Triton X-100 Solubility Assay and Immunoblotting

Exponentially growing cultures were rinsed with PBS and rendered single cell suspensions by incubating with PBS-C-E for 4 min at 37°C, followed by passing once through a 27-gauge needle. Cell concentrations were determined using a particle counter (Coulter Electronics, Inc.). Cell populations were resuspended at 10^7 cells/ml in ice cold lysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl, and 2.5 mM EDTA plus protease inhibitors) containing 1% Triton X-100 (Sigma-Aldrich). Cells were syringed with 10 up and down strokes using a 27-gauge needle, and left at 0°C for 15 min. Soluble fractions were collected after 20 min of centrifugation at 13,500 g at 4°C. The pellets were resuspended in 0.9- μ l lysis buffer without Triton X-100 and syringed 5 times at 0°C with a 23-gauge needle. 0.1 vol 10% SDS was added (equivalent cell concentration: 10^7 /ml) before syringing 5 times with a 23-gauge needle, then 10 times with a 27-gauge needle (pellet fraction). Lysates of soluble and pellet fractions were normalized to cell surface expression level (from 10^3 [NCAM] to 2×10^5 [C1-4L] cell equivalents), resolved by SDS-PAGE, and transferred electrophoretically to a 0.45- μ m PVDF membrane (Millipore) for immunoblotting. The following monoclonal antibodies were used: J22 for CEA, CEACAM6, C-C1S, and C-N; TEC-11 (Dráberova et al., 1997) for C1-4L and C1-C; and ERIC-1 for NCAM, N-C, and N-C blunt at a dilution of 1:1,000 for 1 h at 25°C in TBS-Tween 20/M, and processed as previously described (Screaton et al., 1997). For solubility determinations, increasing volumes of lysates were separated by SDS-PAGE and transferred to PVDF for immunoblotting. Multiple timed ECL exposures of each immunoblot were scanned and analyzed with Adobe Photoshop and NIH Image 1.61 software. Solubility was determined using only values in the linear exposure range.

Adherent Cell PI-PLC Assay

Cells were seeded at 7×10^3 /cm² in 24-well plates in GM on day 0. On day 2, exponentially growing cultures were rinsed twice with PBS, and incubated with 0.03–0.09 U bacterial phosphatidylinositol phospholipase C (PI-PLC; Boehringer Mannheim) in 1:1 DME/PBS plus 0.2% BSA for 40 min at 37°C. The PI-PLC was removed, cultures were rinsed with PBS and rendered single cell suspensions by trituration after incubation with PBS-C-E at 37°C for 4 min. The cells were then processed for FACS analysis as above.

Adhesion Assays

Assays were performed essentially as previously described (Zhou et al., 1993). In brief, 10^6 cells of LR73 transfectant cultures were seeded in 80-cm² culture flasks (Nunc) on day 0. On day 2, cells (CEA family transfectants) were harvested with BACTO-Trypsin (GIBCO BRL) for 3 min at 37°C or with PBS-C-E (N-C blunt). The cell suspensions were syringed up and down once through a 27-gauge needle before cell counting. 3×10^6 cells were resuspended in 3 ml of α -MEM plus 0.8% FBS plus 10 μ g/ml DNase I, and allowed to aggregate at 37°C with stirring at 100 rpm. Aliquots were taken at the indicated time points and the percentage of single cells was determined visually in a hemocytometer.

Colocalization Assay

7×10^3 cells were seeded in 8-well chamber slides (Nunc) on day 0. On day 2, cells were rinsed with PBS and fixed with 4% paraformaldehyde for 20 min at room temperature before placing on ice, or else rinsed and placed on ice immediately to inhibit cellular metabolism and GPI protein mobility. Nonspecific antibody interactions were blocked with DME plus 5% goat serum (DG5) for 20 min before antibody addition. All antibody incubations were carried out for 30 min on ice, followed by 3 \times 5 min rinses with ice-cold DG5. Antibodies and dilutions were as follows: rabbit polyclonal anti-CEA (1:50), goat anti-rabbit-Cy2 conjugate (1:800), ERIC-1 (1:10), and goat anti-mouse-rhodamine conjugate (1:200). For patching, unfixed cells were incubated stepwise with the appropriate primary and secondary antibody combination to stain the first antigen, rinsed with DG5, and incubated at 37°C for 60 min to induce patch formation.

After patching, the cells were returned to ice for staining of the second antigen, as described above. Cells were fixed with 4% paraformaldehyde for 20 min at room temperature after staining, mounted, and viewed with a fluorescence microscope (Nikon).

Results

Synthesis and Characterization of COOH-terminal Domain Exchange Constructs

To determine the domains involved in the inhibition of myogenesis by CEA, chimeric constructs were constructed using CEA and CEACAM1 cDNAs in which the mode of membrane attachment was exchanged. We replaced the TM and cytoplasmic domains of CEACAM1-4L (C1-4L) with the 32 COOH-terminal residues of CEA, which possesses the CEA ω -site for GPI attachment, Ala677, to produce C1-C (Fig. 1). The reciprocal chimera, C-C1S, possesses the complete extracellular domains of CEA attached to the TM and short cytoplasmic domains (43 amino acids total) of the CEACAM1-1S splice variant (Barnett et al., 1993). The COOH-terminal sequence of the CEACAM1-1S (C1-1S) splice variant with its short cytoplasmic domain of about eight residues was selected for use in the construction of C-C1S to most closely mimic a GPI anchor, i.e., a membrane attachment with no intracellular sequence information. Stable pooled transfectant populations or individual clones of L6 myoblasts (see Materials and Methods) expressing the chimeras at their surface were isolated, and the FACS profiles for these and all L6 transfectants used in this study are shown in Fig. 2.

Cell surface proteins that possess a TM domain are soluble in nonionic detergents such as Triton X-100 at 4°C; insolubility under these conditions indicates that the protein may possess a GPI membrane anchor (Brown and Rose, 1992; Hooper and Bashir, 1991; Hooper and Turner, 1988). To determine if the membrane attachment modes were faithfully exchanged in the chimeras, we investigated the solubility of the CEA family members and C1-C and C-C1S in cold Triton X-100 (Fig. 3 A). CEA, CEACAM6, and C1-C detected by immunoblotting and quantitated by

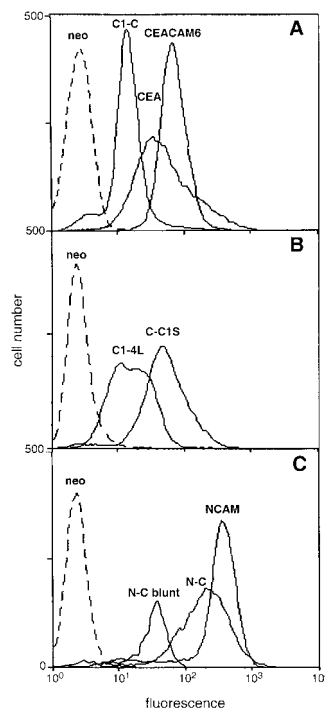


Figure 2. Surface expression of CEA family members, NCAM p125, and chimeric constructs in L6 cell transfectants. FACS profiles showing levels of surface expression of (A) CEA pooled population, CEACAM6 clone, and C1-C pooled population; (B) C1-4L and C-C1S clones; and (C) NCAM, N-C blunt and N-C pooled populations. All cells were labeled with monoclonal anti-CEA or anti-human NCAM antibodies. Immunoreactivity was detected with goat anti-mouse FITC-conjugated antibody, as described in Materials and Methods.

densitometric analysis were ~10–20% soluble, supporting their GPI linkage, whereas C1-4L and C-C1S were >90% soluble, indicating that they both possess a TM anchor. All proteins were >90% soluble in octylglucoside (data not shown), which extracts GPI-linked proteins from Triton X-100-insoluble membrane domains (Brown and Rose, 1992). It was possible that, by overexpressing these GPI-linked proteins, they were saturating the glycosphingolipid rafts in which they are normally localized. However, we observed no change in solubility, for CEA at least, over the range of surface expression found in these transfectants (data not shown). To confirm the presence of a GPI anchor on the various constructs, we also investigated the sensitivity of each protein to bacterial PI-PLC, an enzyme

	EXTRACELLULAR	HYDROPHOBIC/TM
CEA	... ⁶⁵⁸ INLATGRNNSIVKSI ⁶⁷⁷ TVSA	SGTSPGLSAGATVGMIGVLVGVALI*
CEACAM6	... ³⁰⁰ TNSATGLNRTT ³¹⁹ TVTMITVSG	--SAPVLSAVATVGITIGVLRVALI*
C1-4L	... ⁴⁰⁵ PIMLN ⁴⁰⁹ VNYNALPQENGLSPGAIAGIVIGVVALVALIAVALACFLHF ⁴³⁰ GKTGRASD ⁴³⁴ RDLTR...	cyt domain-L
C1-1S	... ⁴⁰⁹ PIMFHVNYNALPQENGLSPGAIAGIVIGVVALVALIAVALACFLHF ⁴³⁴ GKTGSS ⁴³⁸ GPLQ*	cyt domain-S
C-C1S	... ⁶⁵⁸ TGRNNSIVKSI ⁶⁷⁷ TVENGLSPGAIAGIVIGVVALVALIAVALACFLHF ⁶⁹⁹ GKTGSS ⁷⁰³ GPLQ*	cyt domain-S
C1-C	... ⁶⁵⁸ ISK ⁶⁷⁷ NQSDPIMLN ⁶⁹⁶ VSETVSA	SGTSPGLSAGATVGMIGVLVGVALI*
hNCAM ₁₂₅	...SKAAHFV ¹¹⁹ FRTSAQPTAIPA	TLGGNSASYTFVSL ¹³⁸ LFSAVT ¹⁴² LLLLL*
cNCAM ₁₂₀	...SKPAHYAF ¹¹⁹ FRTSAQPTVIPA	TLGSPSTSS ¹³⁸ SVSL ¹⁴² LSAVT ¹⁴⁶ LLLLL*
N-C	... ⁶⁵⁸ KAAHFV ⁶⁷⁷ FRTSAQ ⁶⁹⁶ PSETVSA	SGTSPGLSAGATVGMIGVLVGVALI*
N-C BLUNT	... ⁶⁵⁸ KAAHFV ⁶⁷⁷ FRTSAQPTAIPA	SGTSPGLSAGATVGMIGVLVGVALI*
C-N	... ⁶⁵⁸ SNLATGRNNSIVKSI ⁶⁷⁷ EP-IPA	TLGGNSASYTFVSL ⁶⁹⁶ LFSAVT ⁷⁰⁰ LLLLL*

Figure 1. Amino acid sequence alignment of CEA family members, human (h) NCAM p125, chicken (C) NCAM p120, and chimeras as predicted by their cDNA sequences. The precursor COOH-terminal hydrophobic domains removed by GPI processing are separated by a gap from their extracellular sequences. The amino acid positions given include the leader domain starting with the initiator methionine residue as No. 1. The ω -site for attachment of the GPI anchor, known for CEA and CEACAM6 and inferred for NCAM p125, C1-C, N-C, N-C blunt and C-N constructs, is aligned and denoted by an arrow (see Materials and Methods). The vertical bars represent construct fusion points, and the positions of stop codons are given by asterisks. An I to F mutation created by introduction of a HindIII site at the chimera junction is underlined. The long (-L) and short (-S) cytoplasmic domains of CEACAM1 are denoted by broken underlining.

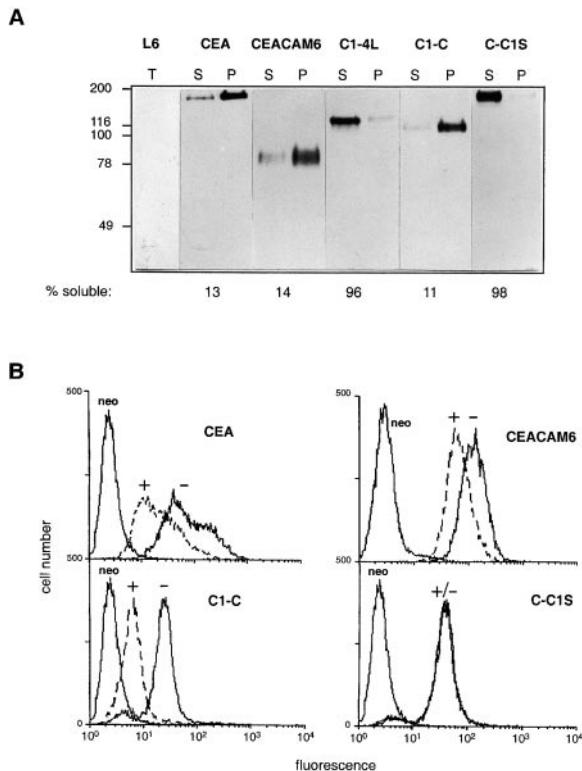


Figure 3. The COOH-terminal domain of CEA confers cold Triton X-100 insolubility and PI-PLC sensitivity on hybrid constructs expressed in L6 transfectants. (A) Soluble supernatant (S) and insoluble pellet (P) fractions of cold Triton X-100 lysates were analyzed by immunoblotting of SDS-PAGE gels. Lysate volumes for each transfectant were normalized to provide an equivalent aggregate densitometric value from soluble and insoluble fractions. All exposures were within the linear range. (B) Sensitivity of cell surface CEA, CEACAM6 (positive controls), C1-C, and C-C1S to PI-PLC (+) or no treatment (-), including vector only controls (neo), as assessed by FACS analysis.

that digests the phosphate-glycerol ester linkage of GPI anchors. Sensitivity of a cell surface molecule to PI-PLC treatment can be measured as a reduction in its cell surface level by cytofluorometry (Lisanti et al., 1990). We observed that the GPI proteins under investigation had different sensitivities to PI-PLC when treated in monolayer culture or in suspension after EDTA or trypsin treatment (data not shown). We therefore chose to treat cells with PI-PLC in monolayer culture to test their sensitivity in a physiologically normal state during their growth phase. L6 transfectants were treated with PI-PLC, labeled with appropriate monoclonal antibodies, and analyzed by FACS (Fig. 3 B). PI-PLC partially removed surface-bound CEA, C1-C, and CEACAM6, confirming their GPI membrane attachment, but did not change surface levels of C-C1S (or CEACAM1, not shown). These results support the conclusion that the C1-C chimera is GPI linked, and that C-C1S possesses a bona fide TM anchor.

Chimeric Constructs Retain Homotypic Adhesion Function

Previous results demonstrated a requirement for intact CEA extracellular adhesion domains to block differentia-

tion (Eidelman et al., 1993). We thus examined the aggregation properties of total transfectant populations of the nonaggregating CHO fibroblast cell line, LR-73, expressing C1-C and C-C1S at their cell surface (Fig. 4 A). Single cell suspensions of all transfectants, including the LR(CEA) and LR(C1-4L) positive controls, aggregated readily at 37°C (Fig. 4 C). Transfectants with the neo vector alone did not aggregate appreciably. The extent of aggregation was greater for C1-C than C-C1S (14 vs. 27% single cells at 2 h), which was probably due to differences in levels of surface expression (FACS mean fluorescence values of 600 vs. 200, respectively). Thus, both C1-C and C-C1S chimeras retained homotypic adhesion function, indicating self-binding of their extracellular domains, and could be used to test for the role of the mode of membrane attachment in inhibiting myogenic differentiation.

Effect of Mode of Membrane Attachment on L6 Differentiation

L6 myoblast transfectants were grown to confluence in growth medium and tested for their ability to differentiate by incubation in low growth factor-containing differentiation medium. Fig. 5 shows photomicrographs of the cultures after 4 d in differentiation medium, at which time both the neo control and L6(C1-4L) cultures showed extensive cell fusion and myotube formation. L6(CEA) cultures, however, consisted of single cells, indicating blocked cell fusion. Interestingly, L6(C-C1S) cultures formed myotubes, whereas cultures of L6 cells expressing the GPI-linked C1-C, even at lower cell surface levels than C-C1S or CEA (Fig. 2), like L6(CEA) remained completely as single cells (Fig. 5 and Table I). This indicates a direct correlation between GPI anchorage of CEA and differentiation-blocking ability.

Effect of Type of GPI Anchor on Adhesion and Differentiation

To confirm the specificity requirements of the extracellular adhesive domains and to test for the necessity of the CEA GPI anchor, chimeric constructs between CEA and the p125 GPI anchored isoform of the more distantly related Ig family member, NCAM, were generated. The COOH-terminal hydrophobic domains and six extracellular amino acids of CEA (as in C1-C) and NCAM were exchanged, to produce N-C and C-N. A construct, N-C blunt, with no extracellular residues from CEA, was also prepared (Fig. 1). Stable pooled transfectant populations

Table I. Differentiation of L6 Transfectants

Cell	% Fusion*
L6(neo)	70
L6(CEA)	0
L6(C1-4L)	84
L6(C-C1S)	60
L6(C1-C)	0
L6(NCAM)	100
L6(NC)	0
L6(NC blunt)	0

*Fusion indices provide a measure of myogenic differentiation and were measured as the % of total cells having three or more nuclei, as described previously (Eidelman et al., 1993).

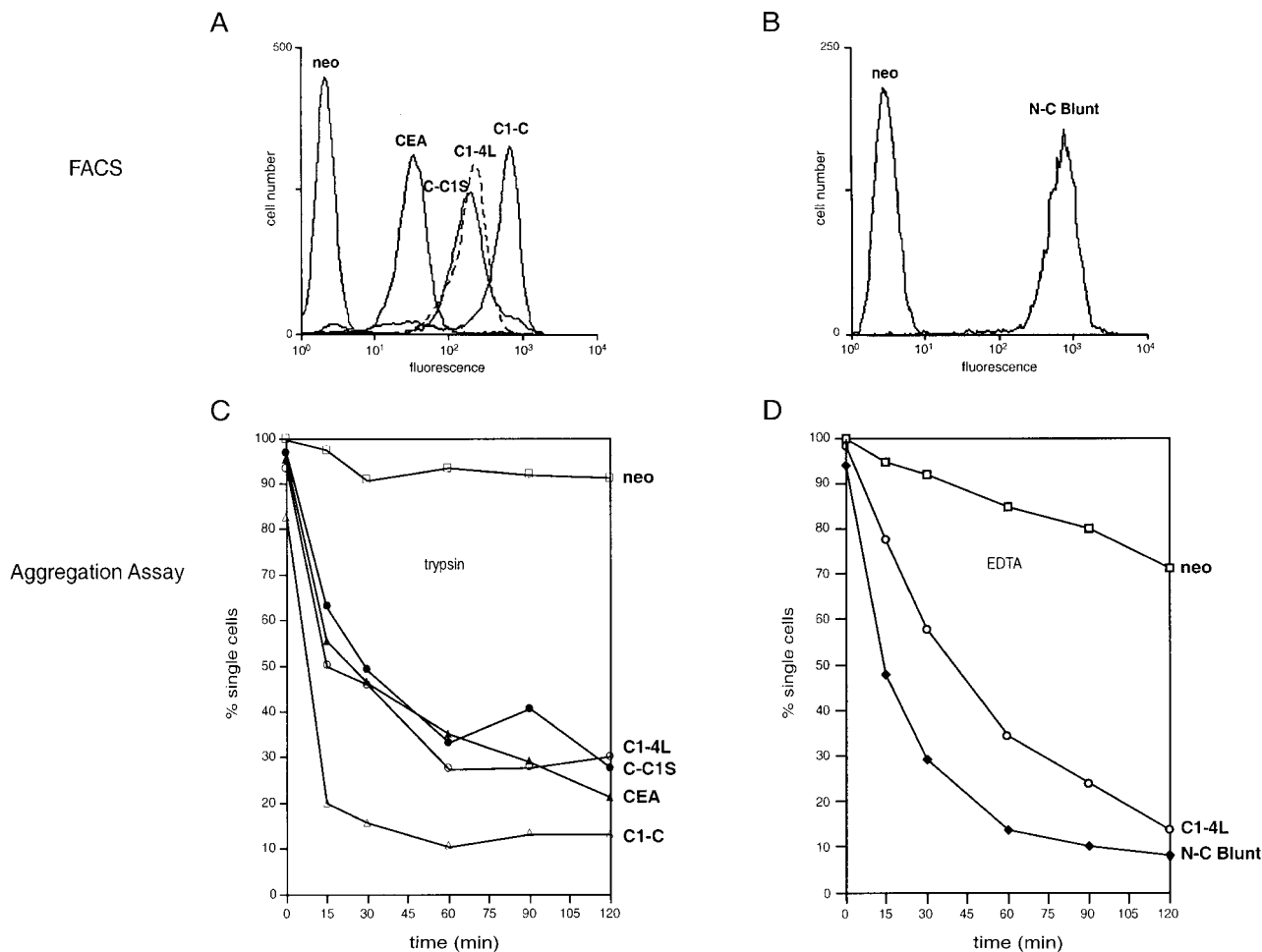


Figure 4. C-C1S and C1-C retain homotypic adhesion function, and NCAM sequences confer homotypic adhesion ability to N-C blunt. (A and B) FACS profiles showing cell surface expression of CEA family members and chimeras in LR73 control cells (neo) and LR73 transfectant cells. (C) Adhesion assay for LR73 control (neo) and transfectant populations by the kinetics of aggregation in suspension. (D) Adhesion assay for LR(N-C blunt) transfectants, harvested with EDTA to avoid proteolysis by trypsin of NCAM sequence, together with neo and C1-4L controls. C-C1S, C1-C, and N-C blunt were pooled transfectant populations, whereas the CEA and C1-4L positive controls were representative clones.

of L6 myoblasts expressing cell surface NCAM and N-C (Fig. 2) and of L6 and LR-73 cells expressing N-C blunt (Figs. 2 and 6) were isolated. No clones showing cell surface expression transfected with the C-N cDNA could be isolated; Western blot analysis and immunofluorescence data demonstrated that the C-N protein was synthesized but accumulated in the ER compartment (data not shown). NCAM, N-C, and N-C blunt were mainly insoluble in cold Triton X-100 (Fig. 6, upper left, ~25% soluble), and sensitive to PI-PLC (Fig. 6), indicating a GPI membrane attachment. Therefore, cold Triton X-100 solubility and PI-PLC treatment do not distinguish biochemically between the GPI membrane anchors of CEA and NCAM.

The adhesion domain of human NCAM has been narrowed to a decapeptide sequence, residues 243-252, present in the third Ig domain, similar to chicken NCAM (Rao et al., 1992; Siu, C.-H., personal communication). We verified that NCAM extracellular sequences retain homotypic adhesion ability when linked to the cell membrane with a CEA GPI anchor by performing an aggregation assay, us-

ing LR-73(N-C blunt) cells together with neo (vector alone) and CEACAM1-4L as negative and positive controls, respectively. Fig. 4 D shows that N-C blunt mediated aggregation of LR fibroblasts efficiently under these conditions (8% single cells at 2 h), indicating that the presence of NCAM Ig domains are sufficient to mediate homotypic adhesion in the chimera.

L6(NCAM), L6(N-C), and L6(N-C blunt) myoblasts were tested for their ability to differentiate by fusion into myotubes in differentiation medium (Fig. 7 and Table I). L6(neo) and L6(NCAM) cells differentiated readily, with a moderate increase in the rate of L6(NCAM) fusion (data not shown). However, L6(N-C) and L6(N-C blunt), like L6(CEA) cultures, were completely blocked in their ability to differentiate, forming no myotubes whatsoever (Table I). The cell surface expression levels of N-C blunt and N-C bracket those of CEA and both are lower than that of NCAM (Fig. 2), so that the differentiation-inhibitory effects of the CEA GPI anchor cannot be ascribed to higher expression levels of the hybrid constructs. These results

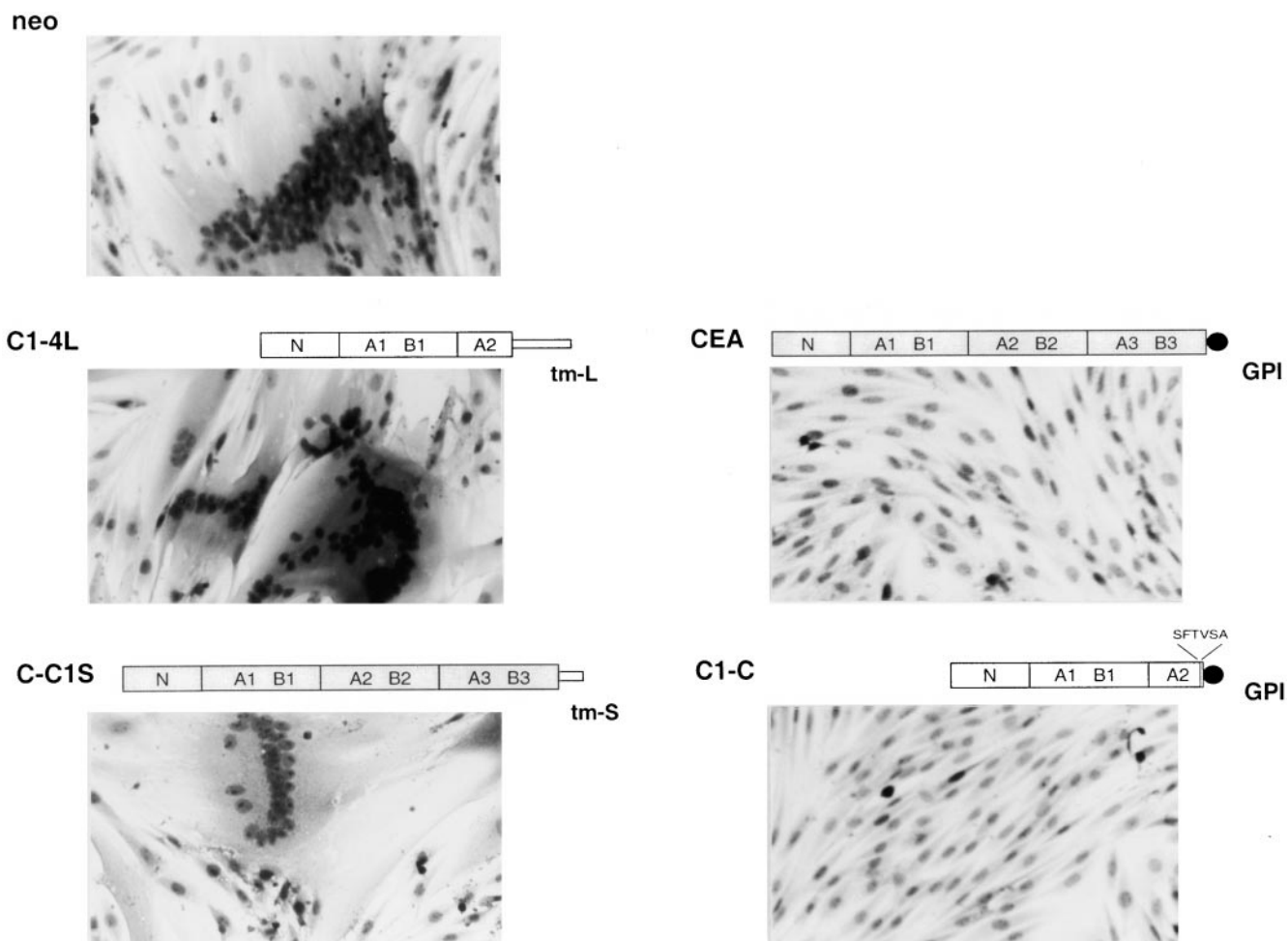


Figure 5. The presence of the GPI anchor–determining COOH-terminal domain of CEA in chimeric constructs is sufficient to inhibit differentiation. Photomicrographs of neo, C1-4L, CEA, C-C1S, and C1-C cultures incubated for 4 d in differentiation medium. C1-4L, with a TM domain, has no effect on differentiation; replacing its TM region with the hydrophobic GPI signal sequence from CEA (C1-C) generates a differentiation-inhibiting protein. Conversely, removing the CEA GPI anchor and replacing it with the TM and cytoplasmic sequences of the short-tail C1-1S isoform abrogates CEA’s differentiation-blocking activity.

confirm that ectopic expression of GPI-linked p125 can accelerate myoblast fusion (Dickson et al., 1990), and demonstrate that NCAM or C1-4L extracellular domains can substitute for those of CEA, and can block fusion, provided they are linked to a CEA-derived GPI anchor. Thus, the specificity of the differentiation block resides in the extreme COOH terminus of CEA.

Cell Surface Distribution of GPI-linked NCAM and CEA

We hypothesized that CEA and NCAM p125, which although both GPI-linked exert opposite phenotypic effects on L6 differentiation, could be distributed differently on the surface of L6 myoblasts as a manifestation of their presumably different GPI anchors. Such a differential distribution might influence their respective molecular interactions, and explain their different biological effects. Therefore, we analyzed the cell surface distribution of CEA and NCAM for overlapping localization in transfectant cells expressing both CEA and NCAM, L6

(CEA+NCAM). In cells fixed with paraformaldehyde before labeling with fluorescent antibodies, both CEA and NCAM showed diffuse, finely punctate staining throughout the cell surface (Fig. 8, a and b), in agreement with previous analyses (Fujimoto, 1996; Mayor and Maxfield, 1995; Harder et al., 1998). Areas of segregation of the two antigens were easily discernible, which suggested that the antigens were not colocalized. To clarify this question, we cross-linked CEA and NCAM one at a time in unfixed L6(CEA + NCAM) cells at 37°C with specific antibodies to consolidate their fluorescent signals into patches, and looked for areas of overlap after patching. If there were no association between the two proteins sufficient to result in colocalization, the steady-state distribution of NCAM should be unaffected by the patching of CEA, and vice versa. After using anti-CEA antibody to cross-link CEA, the CEA fluorescence pattern was no longer diffuse, but concentrated in patches (Fig. 8 c). However, the distribution of NCAM was diffuse and different after patching of CEA (Fig. 8 d). Similar results were seen when the reciprocal experiment was performed (NCAM patched, CEA

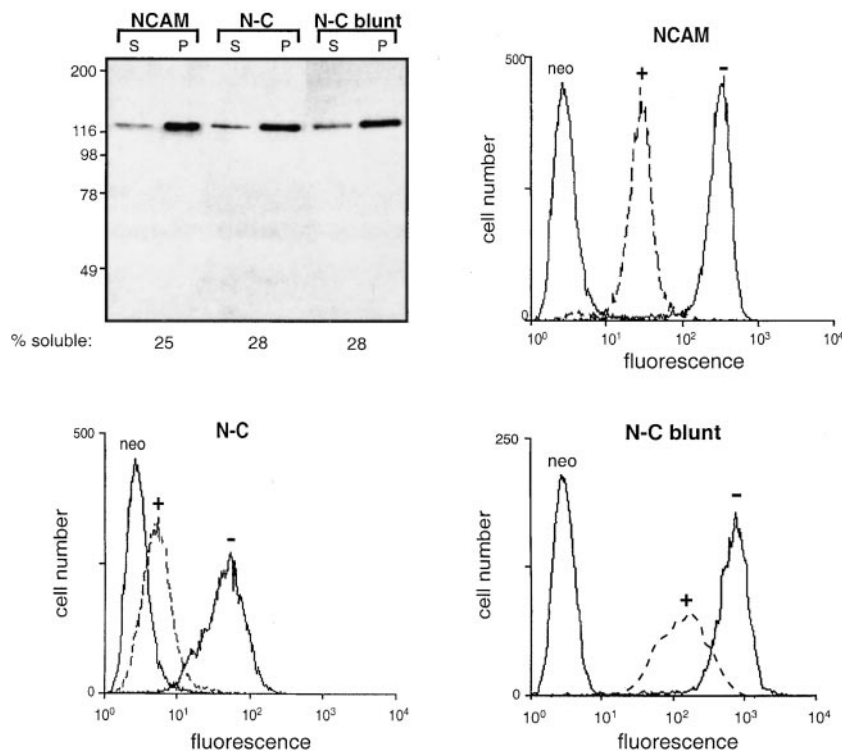


Figure 6. NCAM p125, N-C, and N-C blunt constructs are GPI-linked. (Top left) Immunoblots showing equivalent volumes of cold Triton X-100 soluble (S) and insoluble (P) fractions from L6 transfectants expressing NCAM, N-C, and N-C blunt. Monolayer L6 myoblast cultures producing NCAM and N-C and LR-73 fibroblast cultures producing N-C blunt were treated with PI-PLC before immunostaining with anti-human NCAM mAb, and analyzed by FACS to detect residual cell surface expression. Profiles for control L6 and LR-73 Neo vector-only transfectant cultures are included to indicate levels of background labeling.

unpatched; Fig. 8, e and f). Although patching of one antigen is insufficient to induce copatching of the second, it is possible that both CEA and NCAM are present in the same rafts, but in separate lipid environments within these rafts, which themselves are only weakly associated. This caveat notwithstanding, we conclude that CEA and NCAM are present in different raft domains. This is consistent with their radically different biological effects.

Discussion

Previous work on the structural requirements for the inhibition of myogenic differentiation by CEA showed the necessity of self interaction of the extracellular domains (Eidelman et al., 1993). A GPI-linked adhesion-defective mutant of CEA (Δ NCEA), lacking a large portion of the N domain, did not block differentiation, and the addition of soluble peptides corresponding to the CEA adhesion domains restored myogenic fusion in L6(CEA) cells. Moreover, recent work has shown that single amino acid substitutions in the N domain that affect the adhesion function can abrogate the differentiation blocking activity (Taheri et al., 2000). We subsequently demonstrated that, although both CEACAM6 and all CEACAM1 splice variants also mediate cell aggregation, only GPI-linked CEACAM6, like CEA, could block differentiation, whereas all of the TM-linked CEACAM1 isoforms could not (Rojas et al., 1996). These observations prompted the current investigation of the specific structural features of CEA required to inhibit myogenic differentiation.

The L6 Myoblast System

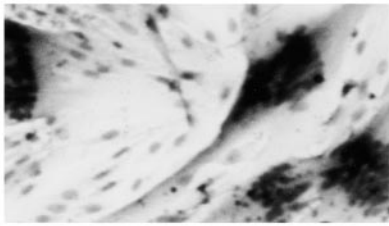
The adequacy of the experimentally convenient L6 myoblast differentiation system as a test system having biologi-

cal relevance deserves comment. The effects of CEA on L6 myoblasts have also been observed in other cellular systems. Thus, CEA disrupts adipogenic differentiation (C3H10T1/2 and 3T3L1 fibroblasts) and neurogenic differentiation (retinoic acid-treated P19 EC cells), and deregulate overexpression (i.e., before polarization and crypt-like formation) both of CEA and CEACAM6 inhibits colonocyte polarization and tissue architecture in vitro and in vivo (DeMarte, L., and C.P. Stanners, unpublished results; Ilantzis, C., L. DeMarte, R.A. Screaton, and C.P. Stanners, manuscript submitted for publication; Malette, B., and C.P. Stanners, manuscript submitted for publication); in addition, anoikis, an apoptotic mechanism of maintaining tissue architecture by killing cells not properly bound to their underlying matrix, is also inhibited in L6 myoblasts and in human colonocytes (Ordoñez et al., 2000). These results indicate that the findings in the L6 model system also apply to more biomedically relevant systems. In further support of this suggestion, we have recently shown that the CEA GPI anchor interacts with a molecular system common to all of these pleiotropic and multiple system effects. Thus, recent evidence indicates perturbation of the function and/or regulation of specific integrin ECM receptors as the underlying cause of the inhibition of both differentiation and anoikis by CEA and CEACAM6 in L6 myoblasts, P19 EC cells, and human colonocytes (Ordoñez, C., R.A. Screaton, C. Ilantzis, M. Fan, L. DeMarte, and C.P. Stanners, manuscript submitted for publication; Malette, B., and C.P. Stanners, manuscript submitted for publication).

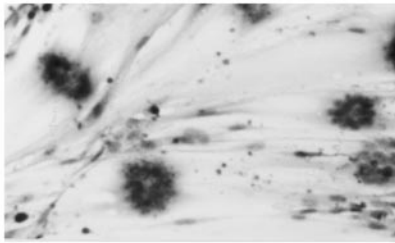
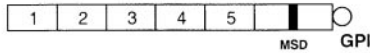
The CEA GPI Anchor Is Required to Inhibit Differentiation

A summary of the results of the domain-exchange experiments between CEA, CEACAM1, and NCAM is shown

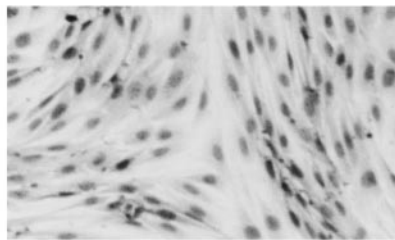
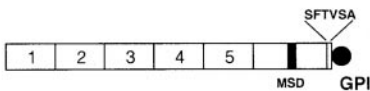
neo



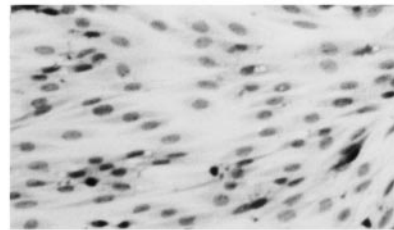
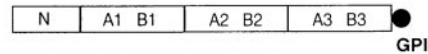
NCAM



N-C



CEA



N-C blunt

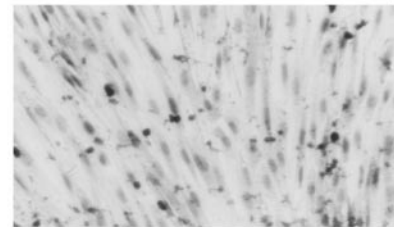
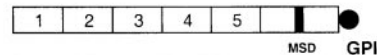


Figure 7. Inhibition of myogenic differentiation requires the specific GPI-determining COOH-terminal domain of CEA. Photomicrographs of neo, CEA, NCAM p125, and N-C and N-C blunt L6 transfectant cultures incubated for 4 d in differentiation medium. Neo and NCAM cultures differentiate into myotubes in differentiation medium, whereas CEA, N-C, and N-C blunt transfectants remain as single cell populations. Thus, acquisition of the COOH-terminal domain of CEA by NCAM p125 converts NCAM into a differentiation-blocking protein.

in Fig. 9. The GPI-linked CEACAM1 chimera, C1-C, inhibited differentiation, indicating that CEA extracellular sequences can be functionally substituted by those of CEACAM1, provided they have the GPI linkage of CEA. Importantly, the reciprocal construct, a TM-linked CEA, C-C1S, allowed fusion to proceed, confirming that the extracellular domains of CEA alone are insufficient to inhibit differentiation and demonstrating that CEA retains this function only when GPI anchored. C-C1S can mediate homotypic intercellular adhesion (Fig. 4), demonstrating that CEA extracellular domains do not require the GPI anchor for self binding.

The p125 isoform of human NCAM is a GPI-linked Ig superfamily member that mediates homotypic intercellular adhesion through its third IgC domain (Siu, C.-H., personal communication). NCAM p125, unlike CEA and CEACAM6, accelerates fusion in L6 myoblasts (Fig. 7 and Screatton, R.A., and C.P. Stammers, unpublished observations), as in C2 myoblasts (Peck and Walsh, 1993), indicating the specificity of the CEA effect. The high degree of

homology between the adhesion domains of CEA and CEACAM1 (>70%) could have accounted for the ability of CEACAM1 extracellular domains to substitute for those of CEA in the C1-C construct. The most highly related amino acid sequences of NCAM and CEA, however, show <30% homology (NCAM 3, 4, and 5, versus CEA B1-A2-B2 IgC domains). Thus, it is intriguing that replacing the COOH-terminal GPI signal sequence of NCAM with that of CEA converts NCAM into a differentiation-inhibiting molecule. NCAM sequences provide a homotypic adhesion function when attached to the CEA GPI signal sequence (Fig. 4), allowing NCAM extracellular domains to substitute for CEA extracellular domains. Given that the extracellular sequences from CEA, CEACAM1, and NCAM can all contribute to the differentiation inhibition phenotype, we conclude that any sequence able to confer homotypic adhesion is required, but that the specificity for the inhibition of differentiation resides in the specific GPI anchor of CEA. By extension, the latter specificity would also reside in the GPI anchor determined by CEACAM6 (Fig. 9).

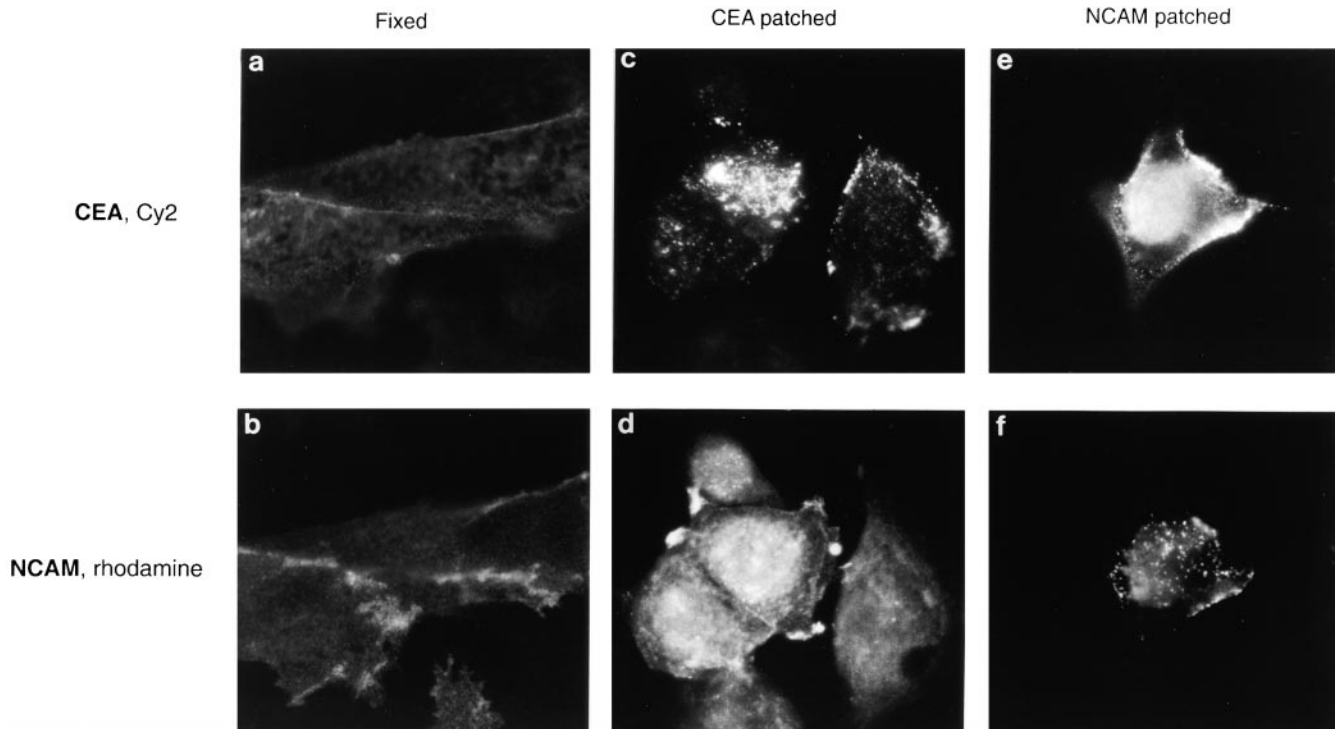


Figure 8. GPI-linked NCAM and CEA are in different plasma membrane domains in L6 transfectants. L6 myoblasts coexpressing CEA and NCAM were analyzed for their surface staining pattern by immunofluorescence after fixation with paraformaldehyde (a and b) or before fixation with (c and d) and without (e and f) patching. Both NCAM and CEA coalesce to form patches when cross-linked with antibodies. However, NCAM is not patched by antibody-induced cross-linking of CEA (c and d), and vice versa (e and f).

GPI Anchors as Gain of Function Structures

Aside from the biological specificity observed here in different GPI anchors derived from different COOH-terminal domains, there is precedent for the fact that the mode of membrane linkage (GPI versus TM) per se can affect protein function. Thus, the cellular isoform of the prion protein requires a GPI signal sequence for conversion to the infectious scrapie isoform; a TM form not targeted to rafts cannot adopt the pathogenic conformation (Kaneko et al., 1997). GPI functional dependency has also been reported for the folate receptor (Wang et al., 1996), murine DAF (Song et al., 1996), and the lymphocyte surface antigens Qa-2 and Ly6 (Robinson et al., 1989; Su et al., 1991). Replacing the GPI anchor of Qa-2 and Ly-6 with a TM sequence abrogates their ability to stimulate T cell activation, whereas replacing the TM domain of the signaling incompetent H-2 protein with a GPI anchor from Qa-2 generates a signaling-competent protein. In some cases, GPI/TM anchor exchange has no measurable effect on function, as shown for CD14 and tissue factor (Lee et al., 1993; Paborsky et al., 1991). Our data provides additional evidence that a GPI membrane attachment can be a gain of function structure.

Membrane Localization May Explain Opposite Effects of TM and GPI CEA Family Members

Extensive evidence suggests that asymmetries in lipid composition in cell surface membranes can affect localization of membrane proteins, exemplified by the mutual af-

finity of sphingolipids/cholesterol found in raft domains and the highly saturated lipid components of the GPI anchor itself (Simons and Ikonen, 1997). We propose that the ability of the GPI-linked CEA family members to inhibit differentiation stems from changes in the repertoire of membrane elements available for interaction due to altered membrane localization. Neither CEACAM1 nor C-1S, both TM proteins, inhibit myogenesis, and both are soluble in cold Triton X-100 (Fig. 3), suggesting that the TM domain guides these proteins into an area of the membrane distinct from the GPI-anchored proteins under investigation. Interestingly, the localization of the TM protein influenza virus hemagglutinin in rafts is dependent on specific amino acid residues found in the exoplasmic half of the membrane-spanning region, residues expected to be in contact with DIG lipids (Scheiffele et al., 1997). However, apical targeting of hemagglutinin in epithelial cells requires these same residues as well as residues towards the COOH-terminal end of the TM domain (Lin et al., 1998).

How does CEA, lacking proteinaceous intracellular domains, transmit information leading to biological responses? The colocalization and coimmunopurification of GPI proteins, second messengers, and *src*-family kinases in membrane rafts, areas which generally exclude TM proteins, has suggested a localization-dependent mechanism whereby GPI proteins may participate in signaling (Brown, 1993; Malek et al., 1994). In hematopoietic systems, cross-linking of GPI proteins results in calcium mobilization, cytokine production/release, and proliferative activity (Rob-

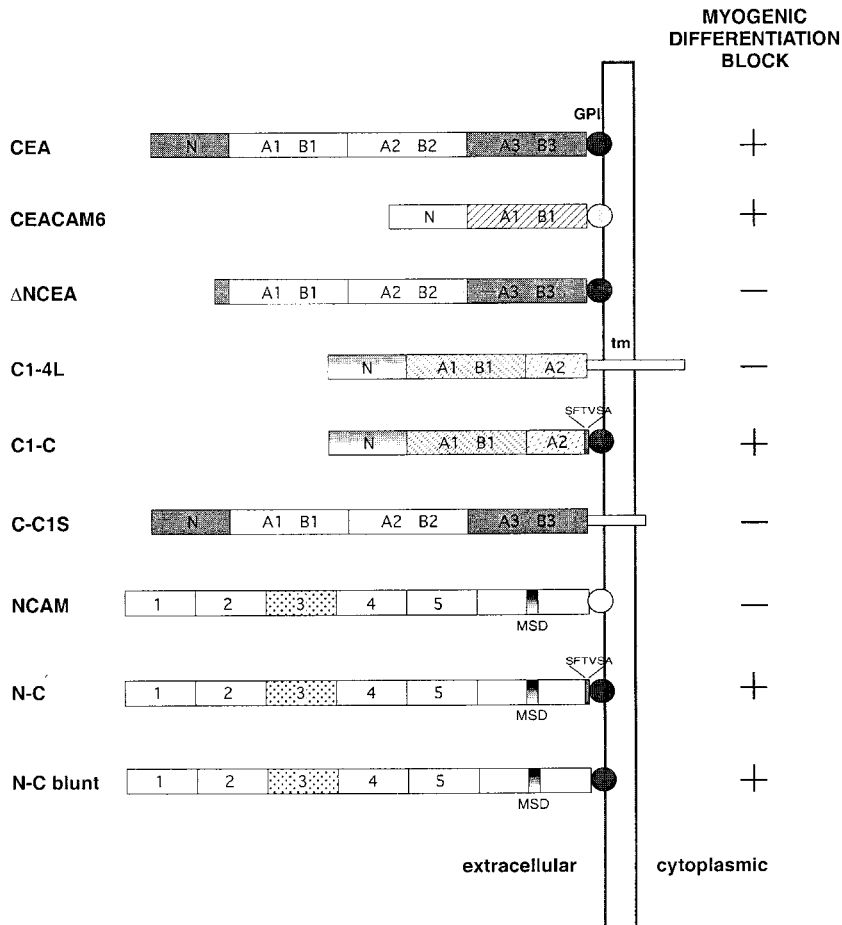


Figure 9. Schematic diagram of CEA family, NCAM 125, and chimeric constructs and their effect on myogenic differentiation. GPI membrane anchors of CEA (black) and NCAM 125 (white) are shown as circles; the six membrane proximal extracellular amino acid residues of CEA that are present in C1-C and N-C are depicted above the construct schematics. The membrane-spanning and cytoplasmic domains of C1-4L and C1-1S (as part of C-C1S construct) are shown as narrow open bars. CEA family member extracellular C2 set Ig domains known to be involved in homophilic adhesion are shown in gray. The NH₂-terminal and internal domains of CEA family members are highly related and are identical in length but are not identical in sequence; this is depicted by different shading. The NCAM (and N-C and N-C blunt) extracellular Ig domains are highly divergent from those of the CEA family; the adhesion domain, Ig3, is dotted.

inson, 1991). Cross-linking of cell surface CEA in rat basophilic leukemia transfectant cells can elicit protein tyrosine phosphorylation of intracellular targets via *src*-family kinases, events which require the GPI anchor (Dräber, P., and C.P. Stanners, unpublished results). The requirement for cross-linking suggests that aggregation of GPI proteins into high molecular weight complexes containing their signaling partners is a prerequisite for signaling, as has been demonstrated for exogenous DAF 'painted' (by exogenous addition) into U937 cell surface membranes (van den Berg et al., 1995).

Specific Raft Domains?

The above considerations pertain to changes in protein function resulting from TM/GPI exchanges. We show here that there exists specificity conferred by the GPI anchor of CEA that is not present in the GPI anchor of NCAM, even where both are attached to the same external domain. How could two proteins, functionally distinguishable only by their GPI anchors, send specific signals, even those eliciting opposite cellular responses? Evidence presented here that CEA and NCAM p125 could be localized to different rafts in L6 cells could be interpreted to indicate that there are distinct raft domains that are able to selectively concentrate different GPI proteins. (As mentioned in Results, this interpretation depends on the assumption that the clustering forces mediated by the antibodies did not pull truly colocalized CEA and NCAM

p125 molecular complexes apart.) A consequence of selective localization would be that CEA, through interactions with signaling components unique to a specific membrane subdomain, could elicit distinct responses from other GPI proteins, e.g., NCAM. Therefore, biological specificity could be determined by differences in the membrane microenvironment in which the GPI anchor is found. The recent demonstration in living cells that GPI proteins exclusively reside in submicron scale domains, ~70 nm in diameter, that contain from 15 to 50 GPI-anchored molecules (Friederichson and Kurzchalia, 1998; Varma and Mayor, 1998), suggests that two different GPI proteins may be found in separate compartments at the cell surface. Contrary to our results with CEA and NCAM, Harder et al. (1998) demonstrated that the GPI proteins PLAP and Thy-1 copatch at the surface of BHK cells when simultaneously cross-linked. However, only variable copatching was seen when one protein was patched alone before staining of the second—a result which was attributed to hindered accessibility of antibody against the second antigen, presumed already to be in the patches. We would otherwise interpret these observations as additional evidence of unique raft domains.

Do Individual GPI Anchors Possess Specific Biological Information?

The ability of the COOH-terminal sequence of CEA to confer the differentiation-blocking activity to CEACAM1

and NCAM poses two questions: first, is there information in the structure of GPI anchors themselves which is capable of conferring specific biological function? Second, how could specific information found in GPI anchors be determined by the COOH-terminal hydrophobic sequence? For the latter, it is possible that the signal sequence at the extreme COOH terminus directs the protein to a specific GPI transamidase activity in the ER, which in turn directs selective modification of the anchor, or that the signal sequence determines the timing of such modifications resulting in changes to their extent. Regarding the former question, GPI anchors vary in the number and specific composition of additional glycan side chains which may be appended to the core anchor glycan structure (Lisanti et al., 1990). Different glycoforms of the GPI anchor on two NCAM isoforms in C2 myoblasts have been found (Mukasa et al., 1995), indicating that different anchor constituents can coexist in the same cell. GPI anchors of DAF and acetylcholinesterase are commonly modified by acylation of the inositol ring, which makes them resistant to PI-PLC treatment (Roberts et al., 1988; Walter et al., 1990). Interestingly, whether or not DAF is inositol acylated depends on the cellular context, suggesting that different cells can attach different anchors to the same protein precursor (Walter et al., 1990). Possibly an unknown substitution (mannosylation, ethanolamine, lipid modification) of the CEA anchor itself could confer specific information. Controlled release of GPI proteins by PI-PLC or PI-PLD would produce free membrane-bound GPIs, which are known to act as hormone-induced second messengers in cell signaling processes (Gaulton and Pratt, 1994). Moreover, a free GPI toxin from the malarial parasite *Plasmodium* initiates a protein tyrosine phosphorylation cascade that induces the upregulation of ICAM1, VCAM1, and E-selectin in endothelial cells (Schofield et al., 1996). The CEA effect on differentiation appears to depend on information provided by the adhesion property of the molecule, however, suggesting that the mechanism of inhibition involves more than a cleavage product of the CEA anchor. Alternatively, the adhesion function may serve to increase the local concentration of CEA before a cleavage event, resulting in a greater impact of the released anchor on signaling. GPI proteins that act as cellular receptors are endocytosed through caveolae, microinvaginations of the plasma membrane, which may serve as focal points to effect a higher local concentration of ligand. In conclusion, there is ample precedent for significant secondary modifications in GPI anchors that could determine which membrane microdomains they associate with, thereby determining their biological specificity.

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