Radical Differences in Functions of Closely Related Members of the Human Carcinoembryonic Antigen Gene Family

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Abstract

The immunoglobulin superfamily represents an ancient, highly diversified group of cell surface and extracellular molecules responsible for a wide range of molecular and cellular recognition functions. The human carcinoembryonic antigen (CEA) subfamily of the immunoglobulin superfamily presents evidence of continuing diversification of the immunoglobulin family, in that some of its members, including CEA itself and nonspecific cross-reacting antigen (NCA), are expressed only in primates and not in rodents. These "new" members are glycoprophosphatidylinositol linked to the external cell membrane and are up-regulated in cancer, unlike members present in both rodents and primates, i.e., biliary glycoprotein (BGP), which are transmembrane linked and down-regulated in cancer. CEA, NCA, and BGP have all been shown to function in vitro as intercellular adhesion molecules. We show here that the properties of adhesion are radically different, in that BGP-mediated adhesion is reversibly Ca2+ and Mg2+ dependent, temperature dependent, and ATP inhibitable, whereas CEA- and NCA-mediated adhesion is the opposite in all aspects. Also, the novel double-reciprocal, antiparallel binding observed for CEA-CEA interactions is not seen for BGP. Finally, the myogenic differentiation block demonstrated for the ectopic expression of CEA in myoblasts was also observed for NCA but not for BGP, which is consistent with the changes in expression seen in cancer. The appearance of new CEA family members with such different properties is discussed in the context of evolution and cancer.

Introduction

The human CEA4 gene family consists of 29 gene sequences that are closely clustered on chromosome 19 (1, 2). These can be divided into two general groups: genes coding for pregnancy-specific glycoproteins and genes coding for the CEA subfamily of cell surface glycoproteins. The latter include CEA itself, NCA, and CGM-6, which are linked to the external cell surface by GPI bonds and BGP, the splice isoforms of which are anchored to the cell membrane by TM domains (Fig. 1). The CEA family represents a subset of the immunoglobulin superfamily (3), with expressed products consisting of a processed leader domain and a variable, immunoglobulin-like, N-terminal domain of 107 amino acids, followed by 0–6 C2-set immunoglobulin-like domains, usually in pairs of 178 amino acids each (A1B1, A2B2, etc.; Refs. 4–9), and terminated by a processed short hydrophobic domain for GPI membrane linkage (10–12) or by a hydrophobic TM domain with a short (9 amino acids) or longer (71 amino acids) cytoplasmic tail (Fig. 1; Refs. 13 and 14). The nucleotide sequence homology of these genes is very high, i.e., higher than 90% in some cases (15), which could indicate a relatively recent replication from a primordial precursor gene in the evolutionary time scale (16). In fact, GPI-linked CEA-like family members have been detected to date only in man and primates (17), whereas rodents seem to possess only the BGP-like TM analogues (18, 19).

Although CEA was discovered in 1965 (20) as a tumor-related antigen that is up-regulated in cancer and has become the major clinical tumor marker for a number of prevalent cancers, its normal function(s) and relevance to malignant transformation were completely unknown until CEA cDNA clones became available (12, 13). Using functional cDNA transfecteds of CHO cells and CEA-producing colorectal carcinoma cell lines, CEA (21), NCA (22, 23), and BGP (24) were then shown to function in vitro, at least, as specific homotypic intercellular adhesion molecules, also capable of heterotypic interactions between themselves (22, 23, 25). The adhesive properties of BGP, however, were found to differ from those of NCA and CEA, in that, although CEA or NCA transfecteds could aggregate in the absence of Ca2+ and at 4°C, aggregation of BGP transfecteds required Ca2+ and physiological temperatures (24).

Adhesion molecules are usually down-regulated in cancer (e.g., E-cadherin; Ref. 26), a phenomenon that can be rationalized by the hypothesis that lower levels of intercellular adhesion molecules would give rise to a looser association.

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4 The abbreviations used are: CEA, carcinoembryonic antigen; NCA, nonspecific cross-reacting antigen; CGM-6, CEA gene family member-6; BGP, biliary glycoprotein; GPI, glycosphinphatidylinositol; TM, transmembrane; CHO, Chinese hamster ovary; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum.
CEA FAMILY

Fig. 1. Structural features of CEA family members involved in this report. Variable immunoglobulin-like, N-terminal domains are shown as linked linear termini, whereas internal C2-set, immunoglobulin-like domains are shown as pairs of loops with disulfide bridges. CEA, NCA, and CGM-6 (M-6) are GPI-linked to the external cell membrane (arrowheads), whereas all BGP splice variants have TM and cytoplasmic domains.

between cells, thus favoring invasion and metastasis. Up-regulation has also been observed, however (e.g., I-CAM in melanomas; Ref. 27), necessitating a different rationalization in these cases. We presented a model for a possible instrumental role of the up-regulation of CEA in the development of colon carcinomas, in which CEA overexpression on the cell surface tended to disrupt cellular differentiation in adult, monolayered, colonic epithelium by the assumption of a multilayered, embryonic-like tissue architecture (21). Consistent with this model, the expression of CEA in rat myo-blasts was shown to block terminal myogenic differentiation and fusion into myotubes, leaving the cells with proliferative potential (28).

CEA and NCA (29, 30) are both overexpressed in cancer; we have recently observed dramatic increases in the expression of these two glycoproteins at the surface of tumor cells by FACS analysis of cell suspensions from human colorectal carcinomas versus adjacent normal tissue. BGP, on the other hand, has been reported to be down-regulated in colorectal carcinomas at the mRNA level (31), although up-regulation of BGP has also been observed (32). Consistent with the former observation of down-regulation, the rat (33) and mouse (34) analogues of human BGP have been shown to act as tumor suppressors.

If CEA and NCA are up-regulated, whereas BGP is down-regulated, in cancer, are there differences in the adhesive and differentiation blocking functions between these molecules, which could help rationalize their opposite correlation with cancer? In this work, we have extended the differences in adhesive properties noted above to include further attributes and, in particular, the fundamental intermolecular interaction involved in adhesion, recently shown for CEA to involve double-reciprocal, antiparallel interactions between two sites on each apposing molecule (35); the latter is shown to be different for BGP. We have also examined the effect of NCA and BGP on myogenic differentiation; consistent with the opposite cancer correlation and the above model, we show that NCA also blocks myogenic differentiation, whereas BGP has no effect.

Results

Differences in Dependence on Ca\(^{2+}\) and Mg\(^{2+}\). We have previously reported that intercellular adhesion mediated by BGP is dependent on the presence of Ca\(^{2+}\) (24) and Mg\(^{2+}\) (36). Oikawa et al. (37) were unable to demonstrate Ca\(^{2+}\)-dependence of BGP-mediated adhesion by their assays, whereas Teixeira et al. (38) showed both Ca\(^{2+}\) and Mg\(^{2+}\)-independent cell intercellular aggregation, as indicated by the rate of disappearance of single cells with time in suspension, whereas BGP-mediated Ca\(^{2+}\)- and Mg\(^{2+}\)-dependent aggregation (Fig. 2). Mg\(^{2+}\) was shown to be reproducibly slightly more effective than Ca\(^{2+}\) in enabling BGP-mediated adhesion in repeated experiments. Thus, in these internally consistent tests, BGP differed from both CEA and NCA in its adhesive requirement for Ca\(^{2+}\) or Mg\(^{2+}\).

Differences in Response to Extracellular ATP. Extra-cellular energy-requiring enzymatic reactions, manifested by a dependence on extracellular ATP, have been implicated in the adhesive activity of other intercellular adhesion molecules (39). In fact, evidence has been reported that rat C-CAM, an analogue of human BGP, could possess an ecto-ATPase activity (40). Therefore, we tested BGP\(_a\) transfectants for sensitivity to externally administered ATP in comparison with NCA transfecents. The results (Fig. 3) showed that 1 mM ATP could completely inhibit adhesion mediated by BGP\(_a\) but had no effect on that mediated by NCA. External ATP also had no effect on the aggregation of CEA transfecents (16; data not shown).

Because ATP can bind Ca\(^{2+}\), it seemed possible that the difference in sensitivity of CEA and NCA transfecents versus BGP transfecents to ATP could have been due to the Ca\(^{2+}\)-dependence of BGP-mediated adhesion. In control experiments, however, the aggregation of LR-73 E-cadherin transfecents, which is exquisitely sensitive to the concentration of Ca\(^{2+}\), was unaffected by the presence of ATP (16).

Externally added ADP or GTP at 2 or 5 m\(M\) had no effect on the aggregation of any of the transfecents, including the BGP\(_a\) transfecents (data not shown). These results thus show a specific inhibitory effect of extracellular ATP on intercellular adhesion mediated by BGP but not by CEA or NCA.

Differences in the Reversibility of Adhesion. We noted a tendency for aggregates of BGP transfecent cells to disaggregate after about 1 h in suspension, unlike CEA or NCA aggregates, which were stable (Figs. 2 and 3; Ref. 24). Therefore, the reversibility of BGP-mediated adhesion was assessed directly by allowing aggregates to form under normal conditions then applying conditions that do not allow aggre-

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Aggregation, such as low temperature, Ca\(^{2+}\) - and Mg\(^{2+}\)-free medium, or ATP-containing medium. The results for temperature reversal are shown in Fig. 4, for which suspensions of both CEA and BGP\(_a\) transfectant cells were incubated for 1 h at 37°C, then shifted to 4°C for 30 min, and finally shifted back to 37°C for the remainder of the incubation. The ready reversibility of the aggregation for the BGP\(_a\) transfectants but not the CEA transfectants is evident. NCA transfectants similarly showed nonreversible aggregation (data not shown). A similar selective reversibility of BGP-mediated aggregation was seen for Ca\(^{2+}\) - and Mg\(^{2+}\)-free medium and ATP-containing medium (16; data not shown). The intermolecular adhesion responsible for BGP-mediated aggregation is, therefore, completely reversible, unlike that mediated by CEA and NCA.

**Differences in Basic Mechanisms of Intermolecular Interaction.** We have shown previously that the intermolecular binding between CEA molecules responsible for mediating intercellular adhesion requires a double-reciprocal interaction between the N-terminal domains and an internal domain, preferably A3B3, of antiparallel molecules on opposed cell surfaces (35). Thus, constructs containing the
CEA N-terminal domain alone or the internal domains alone were shown to be incapable of mediating homotypic intercellular adhesion on their own but were quite capable of mediating heterotypic adhesion when the transfectants were mixed (35). These results have been confirmed recently at the molecular level with purified, complete CEA molecules and CEA domains synthesized in bacteria using direct-binding assays. They have not as yet been extended to NCA transfectants, however. Recently, Teixeira et al. (38) have shown that the N-terminal domain of BGP can bind to BGP\(\alpha\) (Fig. 1), thus proving that the N-terminal domain is involved in intermolecular binding, and that BGP molecules with the short cytoplasmic tail are competent in adhesion. These findings did not, however, distinguish between N-N and N-terminal domain interactions as being responsible for BGP-mediated adhesion.

To discern which of these intermolecular binding mechanisms BGP uses, LR-73 transfectants of two splice variants, BGP\(\alpha\) and BGP\(\alpha\), consisting of the N-terminal domain alone linked directly to the TM domain with long and short cytoplasmic tails, respectively (Fig. 1), were tested in aggregation assays. The results (Fig. 5) indicate that, unlike CEA, the BGP N-terminal domain alone is capable of mediating homotypic intercellular adhesion. Furthermore, the observed aggregation shows the normal BGP requirement for Ca\(^{2+}\) and physiological temperatures (Fig. 5); all of the other particular features of BGP-mediated adhesion, such as reversibility and ATP sensitivity, are also shown by BGP\(\alpha\) (16). Thus, the N-terminal domain alone of BGP is necessary and sufficient for mediating intercellular adhesion, unlike CEA. In addition, the N-terminal and TM domains are all that are required to show the particular adhesive properties of BGP; the internal domains and the length of the cytoplasmic tail are irrelevant. These results do not, however, rule out the possibility of N-terminal domain interactions in addition to the N-N interactions shown here; experiments are in progress to address this issue.

**Differences in Effects on Myogenic Differentiation.** We reported previously that the cell surface expression of CEA can completely block myogenic differentiation of L6 rat myoblasts, an effect that can be reversed by the addition of adhesion-blocking peptides representing the N-terminal or A3B3 domains (28). The question of whether NCA and the splice variants of BGP can also block myogenic differentiation was addressed by the isolation of stable transfectants of L6 cells producing comparable cell surface levels of these CEA family members. Both morphological (i.e., fusion into multinucleated myotubes) and biochemical (i.e., production of myosin) differentiation were, like CEA, completely blocked by NCA but not by BGP\(\alpha\) (Fig. 6). Quantitative results, i.e., fusion indices, for two independently isolated BGP\(\alpha\) transfectant clones, a total stable BGP\(\alpha\) transfectant population, and a BGP\(\alpha\) clone are shown in Table 1. All demonstrated a relatively high degree of myogenic fusion equal to that of the parental L6 cells. Finally, the kinetics of fusion, shown in Fig. 7, was approximately the same for parental L6, a BGP\(\alpha\) transfectant clone, and a BGP\(\alpha\) transfectant clone, whereas the NCA transfectant clone showed no fusion whatsoever over a period of 9 days. These results show that BGP has no effect on the differentiation of myoblasts, which is radically different from the blocking effect of CEA and NCA.

**Discussion**
The above results show that the human CEA family members CEA and NCA have markedly different biological properties from the closely related member BGP, and that these differences could provide a rationale for their opposite correlation with cancer. Thus, CEA and NCA mediate homotypic intercellular adhesion, which is relatively insensitive to environmental conditions, whereas BGP mediates reversible adhesion, which requires Ca\(^{2+}\) or Mg\(^{2+}\) and physiological temperatures and which can be blocked with externally administered ATP. Even the basic intermolecular binding mechanisms differ for CEA and BGP, in that CEA-CEA interactions require double-reciprocal binding between the N-terminal and A3B3 domains of antiparallel molecules, whereas BGP-BGP interactions can occur through the N-terminal domains alone. Finally, CEA and NCA block myogenic differentiation completely, consistent with the observed up-regulation of the cell surface levels of these molecules in cancer (29, 30), whereas BGP has no effect, consistent with the down-regulation recently observed for this molecule in colorectal cancer (31) and the demonstration of a tumor suppressor role for rodent BGP (33, 34). In recent experiments analyzing purified single-cell suspensions of colonic epithelial cells from human

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\(^{6}\) A. Fuchs and C. P. Stanners, unpublished observations.
colon carcinomas of various grades and states of differentiation and from adjacent normal epithelium, we have in fact observed a positive correlation between the cell surface levels of CEA and NCA in FACS distributions and tumor stage; i.e., for a series of well-differentiated to less well-differentiated tumors, the more advanced the stage and the less differentiated the carcinoma, the higher the levels of these CEA family members. The myogenic differentiation system thus may provide a valid model system for events occurring during colonic carcinogenesis.

We have reported previously that BGP-mediated intercellular adhesion is dependent on Ca\(^{2+}\) (24), whereas this de-
Table 1. Differentiation of L6 BGP transfectant clones

<table>
<thead>
<tr>
<th>Cells*</th>
<th>FACS mean valueb</th>
<th>Fusion indexc</th>
</tr>
</thead>
<tbody>
<tr>
<td>L6</td>
<td>3</td>
<td>0.74-0.85</td>
</tr>
<tr>
<td>L6 (CEA)</td>
<td>64-120</td>
<td>0</td>
</tr>
<tr>
<td>L6 (BGPa)-11</td>
<td>56</td>
<td>0.81</td>
</tr>
<tr>
<td>L6 (BGPa)-10</td>
<td>45-67</td>
<td>0.84</td>
</tr>
<tr>
<td>L6 (BGPa)-total</td>
<td>44</td>
<td>0.84</td>
</tr>
<tr>
<td>L6 (BGPa)-3</td>
<td>63</td>
<td>0.90</td>
</tr>
</tbody>
</table>

* Parental L6 myoblasts, CEA transfectant clones, BGPa, or BGPb transfectant clones (denoted by numbers), and a BGPa total transfectant population (see "Materials and Methods") were subjected to assays for myogenic fusion.

Surface expression of CEA family members was assessed by FACS as outlined in "Materials and Methods." Mean values of the resultant distributions are shown. The range of values refers to FACS mean values obtained in different experiments.

Fusion indices were determined, as indicated in "Materials and Methods," after 8 days in differentiation medium.

Dependence could not be demonstrated by Oikawa et al. (37). Subsequent work has shown that the effect can depend on the level of BGP production on the cell surface, because cell aggregation mediated by very low and very high levels of surface BGP showed less or no Ca2+ dependence on intercellular adhesion. It is also possible that the differences in results could be ascribed to the variable presence of other divalent cations, because Mg2+ can also affect BGP-mediated adhesion (Fig. 2; Ref. 36 and 38). In any event, these results show that the Ca2+ dependence of cellular aggregation seen for BGP, which is unusual for an immunoglobulin family member, is not of the same type as that observed for the cadherin family of intercellular adhesion molecules, in which Mg2+ cannot substitute for Ca2+ (41). The effect on BGP could be due to divalent, cation-induced changes in the conformational state of these molecules, which could also be involved in their observed sensitivity to temperature and reversibility of homotypic adhesion. Alternatively, all of these effects could be ascribed to indirect effects, such as clustering of BGP molecules on the cell surface, or to "inside-out" modulation of the adhesive strength of BGP interactions mediated by cellular structures.

The same speculations could be applied to the inhibition of intercellular adhesion observed for the administration of ATP to BGP transfectants but not to CEA or NCA transfectants; the effect could be due to selective modulation of the conformation of BGP versus CEA and NCA molecules, perhaps through the activity of an ATP-requiring extracellular enzyme (40), or the effect could represent the consequence of some cellular event, such as phosphorylation of tyrosine residues in the cytoplasmic domain of BGP (42, 43). Such events could not occur for the GPI-linked CEA and NCA, which lack cytoplasmic domains. More trivial explanations involving, e.g., changes in the charge of the extracellular milieu, are rendered unlikely by the fact that the phenomenon was not observed for ADP or GTP.

The immunoglobulin superfamily of genes appeared very early in evolution and has evolved to embrace an unusually broad repertoire of functions. Is there any reason to believe that this diversification has stopped? The CEA gene subfamily could represent an example of continuing evolution of one of the immunoglobulin superfamily branches; its close clustering on one chromosome in the human genome (1), the high degree of homology between the nucleotide sequences of its members (15), the fact that the nucleotide sequences are more closely aligned than derived amino acid sequences (4, 16), and the fact that CEA and NCA have been found in primates (17) but not in rodents (18, 19) all point to the recent and presumably continuing evolution of the CEA family. If the view is held that molecular evolution occurs by the replication of genes, in which an original beneficial function is held by the parental gene and new beneficial functions are "sought" by slowly diverging copies, the recent emergence of copies such as CEA and NCA with markedly different adhesive properties and effects on the phenotypes of cells presents a particular difficulty: how can radical functional differences be quickly absorbed by complex, integrated biological systems? In fact, the addition of the human CEA gene (44) or even SV40 promoter-driven CEA cDNA (45) to mice by transgenesis is without apparent effect on the phenotype, despite both embryonic and adult expression of CEA in various tissues. This may attest to the inherently high plasticity of multicellular organisms in their ability to retain overall phenotypic integrity in the face of rather drastic changes in the functions of individual components determined by elements in the germ line. Of relevance to carcinogenesis, however, genetic changes in somatic tissue with

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more “brittle” gene expression programs may not be tolerated as readily, as witnessed by the complete inhibitory effect of CEA or NCA expression on the differentiation of myoblasts, an effect in the direction of malignant transformation. Whether this effect actually occurs in vivo and whether it can be extended to other cases of differentiation is currently under investigation. If so, the question of its utility for the organism, resulting in retention during evolution, arises. Explicit (rather than consequential) inhibition of differentiation, which could be useful during embryonic morphogenesis in which cell collectives need to develop a tissue architecture before differentiation, comes to mind. Experiments are in progress using systems with variable levels of differentiative capacity to test this speculation.

Materials and Methods

Cell Lines and Transfectants. The LR-73 line (46) of CHO cells and derived transfectants were grown in monolayer culture at 37°C in a humidified atmosphere 10% air plus 5% CO₂ using α-MEM (47) containing 10% FBS as growth medium. The L6 line (28) of rat myoblasts was maintained as above in subconfluent culture (to prevent selection for nonfusing variants) in DMEM plus 10% FBS (28).

Stable transfectant clones of LR-73 cells expressing CEA and BGP₃ (denoted BGP₃I in Ref. 24) were obtained and characterized as described previously (24). The particular NCA transfectant clone used in these experiments was characterized previously (23). BGP₃ and BGP₄ (48) transfectant clones were obtained by calcium phosphate coprecipitation with cDNA expression constructs and pSV2Neo(49), followed by selection of G-418-resistant clones (24). Expression was assessed by FACS analysis of cell suspensions using rabbit anti-CEA polyclonal antibody, as described previously (28), and clones with cell surface expression approximately equivalent to that of the above NCA, CEA, and BGP₃ transfectants were selected for study. These CEA family members are sufficiently similar that the polyclonal anti-CEA antibody used reacts well with all of them; the possible presence of some components in the polyclonal antiserum directed against epitopes present only on CEA would tend to underestimate, if anything, the cell surface levels for the other CEA family members.

Stable transfectants of L6 cells expressing comparable surface levels (as assessed by FACS analysis) of CEA, NCA, BGP₃, and BGP₄ were isolated (28), except that a total stable transfectant population was also obtained for BGP₃ by FACS and culturing the high surface BGP-expressing cells of the total G-418-resistant population.

Cell Aggregation Assays. The kinetics of the disappearance of single cells, due to the formation of clumps, was used as a measure of intercellular adhesion and was carried out in α-MEM or Puck’s saline, with or without Ca²⁺ and Mg²⁺, exactly as described previously (24). α-MEM contains 2 mM Ca²⁺ and 0.83 mM Mg²⁺ (49).

For experiments examining the effect of ATP and other nucleotides on aggregation, ATP was added to the aggregation medium (α-MEM, 0.8% FBS, and 50 μg/ml DNase) at 0.5, 1.0, or 2.0 mM prior to resuspending the cells for the aggregation assay. Because the results were very similar at all three concentrations, data for only 1.0 mM are presented.

Assays for Myogenic Differentiation and Fusion. The ability of L6 myoblasts and derived transfectants to fuse into myotubes was assessed by seeding cultures of 10⁵ cells/cm² in 35-mm plastic dishes in culture dishes in growth medium. Three days later, the medium was changed to differentiation medium (DMEM and 2% horse serum), as described (28). After incubation for 3–5 days, cultures were fixed and stained with H&E, and the number of nuclei in cells with more than three nuclei per cell expressed as a percentage of the total nuclei was determined by light microscopy.

A measure of myogenic differentiation at the biochemical level was obtained by staining cultures treated as above to induce morphological differentiation with antimyosin monoclonal antibody (50) and FITC-conjugated, antimouse IgG.

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References


