

CDO: An Oncogene-, Serum-, and Anchorage-regulated Member of the Ig/Fibronectin Type III Repeat Family

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Abstract. Cell adhesion molecules of the Ig superfamily are implicated in a wide variety of biological processes, including cell migration, axon guidance and fasciculation, and growth control and tumorigenesis. Expression of these proteins can be highly dynamic and cell type specific, but little is known of the signals that regulate such specificity. Reported here is the molecular cloning and characterization of rat CDO, a novel cell surface glycoprotein of the Ig superfamily that contains five Ig-like repeats, followed by three fibronectin type III-like repeats in its extracellular region, and a 256-amino acid intracellular region that does not resemble other known proteins. In rat embryo fibroblasts, *cdo* mRNA expression is maximal in confluent, quiescent cells. It is rapidly and transiently down-regulated by serum stimulation of such cells, and is constitu-

tively down-regulated in oncogene-transformed derivatives of these cells. CDO protein levels are also dramatically regulated by cell-substratum adhesion, via a mechanism that is independent of *cdo* mRNA expression. The amount of CDO produced at the surface of a cell may therefore be governed by a complex balance of signals, including mitogenic stimuli that regulate *cdo* mRNA levels, and substratum-derived signals that regulate CDO protein production. *cdo* mRNA is expressed at low levels in most adult rat tissues. A closely related human gene maps to chromosome 11q23–24, a region that displays frequent loss of heterozygosity in human lung, breast, and ovarian tumors. Taken together, these data suggest that loss of CDO function could play a role in oncogenesis.

CELL adhesion molecules (CAMs)¹ of the Ig superfamily (IgSF) are implicated in cell-cell interactions that mediate, in part, a wide variety of developmental, physiological, and pathological processes (for reviews see Edelman and Crossin, 1991; Hynes and Lander, 1992; Brummendorf and Rathjen, 1995; Cunningham, 1995). In general, CAMs of the IgSF mediate Ca²⁺-independent, homo- and heterophilic cell-cell adhesion. These molecules typically possess an extracellular region that consists of several Ig-like domains followed, in many cases, by a number of fibronectin type III (FNIII)-like domains. Members of the IgSF that contain both Ig-like and FNIII-like repeats (e.g., neural cell adhesion molecule (N-CAM), L1, F11/contactin, etc.) have been mainly im-

pllicated in developmental processes in the nervous system, including cell migration, neurite extension, and axon guidance and fasciculation (Hynes and Lander, 1992; Tessier-Lavigne and Goodman, 1996).

Cell-cell recognition also plays an important role in the proliferative capacity of cells both inside and outside the nervous system, and Ig/FNIII family members may be involved in this phenomenon as well. For example, cell surface N-CAM mediates contact inhibition of growth of a mouse fibroblast cell line (Aoki et al., 1991), and addition of soluble N-CAM to primary cultures of rat astrocytes specifically inhibits their proliferation (Sporns et al., 1995).

Consistent with a role for CAMs in controlling cell proliferation, a large body of evidence suggests that alterations in adhesion-mediated cell regulation may play a role in induction and/or maintenance of the neoplastic phenotype (for review see Hedrick et al., 1993). It has been known since the 1940s that cancer cells display altered adhesiveness when compared to their normal counterparts (Coman, 1944). Moreover, malignant tumors exhibit a loss of appropriate tissue architecture, altered cell shape, cell crowding, and invasiveness. Tumor cell lines, or cells transformed in vitro, show a loss of contact inhibition of growth and proliferate in an anchorage-independent

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1. *Abbreviations used in this paper:* CAM, cell adhesion molecule; C-CAM, cell-cell adhesion molecule; FNIII, fibronectin type III; N-CAM, neural cell adhesion molecule; IgSF, Ig superfamily; LOH, loss of heterozygosity; FNIII, fibronectin type III.

fashion. Although the molecular bases of these phenotypes are still poorly understood, they may arise, in part, because of loss of expression or mutational inactivation of genes that encode CAMs (Hedrick et al., 1993; Berx et al., 1996).

A candidate tumor suppressor gene, *DCC*, encodes a member of the Ig/FNIII family containing four Ig-like repeats, and six FNIII-like repeats in its extracellular region (Fearon et al., 1990; Hedrick et al., 1994; for reviews see Fearon and Pierceall, 1995; Fearon, 1996). *DCC* was isolated by positional cloning in a region of chromosome 18q that commonly displays loss of heterozygosity (LOH) in colorectal cancers. *DCC* was included in the region of allelic loss in >90% of colorectal cancers that exhibited LOH of 18q. Furthermore, loss of *DCC* protein expression in colorectal tumors is a negative prognostic marker of survival rate in patients with stage II and stage III disease (Shibata et al., 1996). Additional studies of *DCC* expression and allelic loss indicated that this gene may also be involved in many additional types of cancer and may play a role in cell differentiation (Fearon, 1996). *DCC* was also recently shown to function as an evolutionarily conserved receptor or component of a receptor for netrin-1, a secreted and membrane-associated protein that acts as a guidance cue for migrating axons in the developing nervous system (Chan et al., 1996; Keino-Masu et al., 1996; Kolodziej et al., 1996). These data raise the possibility that loss of *DCC* function could alter the motility of cancer cells.

An additional IgSF member implicated in carcinogenesis is cell-cell adhesion molecule (C-CAM)/biliary glycoprotein, which contains four Ig-like domains in its extracellular region. Expression of the *C-CAM/Bgp-1* gene was down-regulated in colorectal and prostate malignancies (Kleinerman et al., 1995; Neumaier et al., 1993), and ectopic restoration of its expression suppressed tumorigenicity of cell lines derived from such tumors (Hsieh et al., 1995; Kunath et al., 1995).

It is clear from the studies cited above that CAMs of the IgSF play a role in coordinating normal cellular architecture, motility, and growth control, and that loss of function of such molecules may play an important role in multi-stage carcinogenesis. We have previously developed and characterized a panel of somatic cell mutant lines derived from Rat 6 embryo fibroblasts that are specifically resistant to the induction of anchorage-independent growth by various oncogenes (Krauss et al., 1992; Feinleib and Krauss, 1996; Kang and Krauss, 1996). Because the oncogene-resistant phenotype of these cells was dominant in somatic cell hybridizations, we sought to isolate genes whose expression is up-regulated in the mutant cell lines relative to a transformation-sensitive control cell line. Reported here is the molecular cloning and characterization of a rat gene designated *cdo* (C-CAM-related/down-regulated by oncogenes), which encodes a novel transmembrane member of the Ig/FNIII family. In rat fibroblasts, *cdo* expression is maximal in confluent, quiescent cells. *cdo* mRNA and protein levels are strongly, but transiently, reduced when such cells are stimulated to reenter the cell cycle and are constitutively down-regulated in oncogene-transformed derivatives of these cells. Furthermore, CDO protein levels are strikingly regulated by cell-substratum adhesion, with a

mechanism that is independent of *cdo* mRNA expression. A closely related human gene maps to chromosome 11q23–24, a region that displays LOH in human lung, breast, and ovarian tumors. Taken together, these data suggest that loss of CDO function might play a role in oncogenesis.

Materials and Methods

Cell Culture

All Rat 6–derived cell lines were routinely maintained in DME plus 10% bovine calf serum, as previously described (Krauss et al., 1992). NIH 3T3 and NIH 3T3/*ras* cells were cultured in DME plus 5% FBS. Soft agar assays and preparative methylcellulose cultures were performed as previously described (Kang and Krauss, 1996). Cells were harvested from preparative methylcellulose cultures 48 h after inoculation into the semisolid medium, and were >98% viable as assessed by trypan blue dye exclusion and replating assays (Kang and Krauss, 1996). For serum stimulation experiments, confluent cultures of Rat 6 cells were washed twice with PBS, and fed with DME plus 0.1% calf serum. After 48 h, the cultures were refed with DME plus 20% calf serum and harvested for analysis at various time points thereafter.

RNA Isolation, Library Construction, and Screening

RNA used for cDNA library construction was prepared by the guanidine thiocyanate/cesium chloride centrifugation method (Chirgwin et al., 1979), and the poly(A)⁺ fraction was isolated by oligo(dT)-cellulose chromatography (Aviv and Leder, 1972). RNA used for Northern blot analyses was isolated either as described above or with the TRIzol reagent (GIBCO BRL, Gaithersburg, MD), which was used according to the manufacturer's instructions.

An oligo(dT)-primed cDNA library was constructed in λ ZAPII (Stratagene, La Jolla, CA) with mRNA isolated from the transformation-resistant, mutant cell line, R-1a (Krauss et al., 1992). The library was constructed with the SuperScript Choice system kit from GIBCO BRL, and was screened by differential hybridization (Johnson et al., 1987) with ³²P-labeled cDNA prepared from R-1a and control, transformation-sensitive PKC3-F4 cells. One clone that exhibited a greater signal with R-1a–derived cDNA, relative to PKC3-F4–derived cDNA, represented a partial cDNA for *cdo*. Clones representing a nearly full-length cDNA were isolated from a random-primed λ ZAPII library constructed with mRNA from Rat 6 cells. A human cDNA that is closely related to rat *cdo* was isolated by screening human fetal lung and fetal brain cDNA libraries (obtained from A. Chan and S. Aaronson, Mount Sinai School of Medicine; and Clontech, Palo Alto, CA, respectively) with the full length rat *cdo* cDNA.

DNA Sequencing and Computer Analysis

DNA sequencing was carried out by a combination of manual and automated methods. Manual sequencing was performed with a Sequenase 2.0 kit (United States Biochemical Corp., Cleveland, OH). Automated sequencing was performed at the Mount Sinai Molecular Biology Core Facility on an Applied Biosystems 373 computerized DNA sequencer (Foster City, CA) or at the Biotechnology Center at Utah State University (Logan, UT). Searches of the DNA databases were performed with the BLAST server service, and sequence analysis and alignments were carried out with MacVector (International Biotechnologies, Inc., New Haven, CT) and GCG software (Genetics Computer Group, Madison, WI).

RNA Analyses

Northern blot analyses were performed by fractionating total cellular or poly(A)⁺ RNA through agarose/formaldehyde gels, blotting to nylon membranes, and hybridizing with DNA probes as described by Krauss et al. (1992).

For the study of *cdo* expression in adult rat tissues, PCR-based “exon-connection” assays were carried out (Fearon et al., 1990). A portion of the *cdo* gene was isolated from a rat genomic library (a gift of S. Salton, Mount Sinai School of Medicine) and a partial exon/intron structure deduced over a ~15-kb region. Random primers and reverse transcriptase were used to generate cDNA from total RNA derived from rat tissues. PCR primers (5'-CACACAGTCAGAAGCGTTCCTC-3' [sense], and 5'-

AGGATAAGAGGCTACCACTGGG-3' [antisense]) directed against sequences from separate exons were then used to amplify a cDNA product that linked these exons. PCR reactions were fractionated on agarose gels, blotted to nylon membranes, and probed with a *cdo* cDNA probe. To analyze differential splicing of the fifth Ig repeat of *cdo* in Rat 6 cells, reverse transcription-PCR was performed on total Rat 6 cell RNA with the following primers: 5'-GGAAGCAACTGGAGAAAGG-3' (sense) and 5'-GTCTCGTTCATCGTTCTG-3' (antisense). The products were analyzed by agarose gel electrophoresis and ethidium bromide staining.

Flow Cytometry

Cells were stained with propidium iodide buffer (50 µg/ml propidium iodide, 0.1% sodium citrate, 0.1% Triton X-100) in the presence of 10 µg of DNase-free RNase and analyzed by flow cytometry on a fluorescence-activated cell sorter (FACS[®]can; Becton and Dickinson, Co., Mountain View, CA). The data were analyzed by the CellFIT software program (Becton and Dickinson, Co., Mountain View, CA).

Antibodies and Western Blot Analyses

A portion of the intracellular region of rat *cdo* (amino acids 1057–1241) was ligated into pGEX-5X-1 (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) to produce a glutathione-S-transferase fusion protein. Fusion protein produced in *Escherichia coli* was purified by SDS-PAGE and electroelution. Rabbit polyclonal antibodies to CDO protein were generated with a proprietary immunization regimen referred to as PolyQuik[™] (Zymed Laboratories Inc., South San Francisco, CA). Crude antisera were used for Western blot analyses.

Immunoblot analyses were performed essentially as described in Kang et al. (1996). Cells from monolayer or methylcellulose suspension cultures were harvested in radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-HCl, pH 7.2, 150 mM NaCl/1% Triton X-100/1% sodium deoxycholate/0.1% SDS/1 mM EGTA) containing 1 mM PMSF, 10 ng/ml leupeptin, 50 mM NaF, and 1 mM sodium orthovanadate. Total proteins were then separated by SDS-PAGE, transferred to nitrocellulose membranes (Amersham Corp., Arlington Heights, IL), and probed with anti-CDO antiserum. The blots were washed and reprobed with HRP-conjugated secondary antibody and specific protein bands visualized with the ECL chemiluminescent detection system (Amersham Corp.) as described previously (Kang et al., 1996).

Surface Labeling and Tunicamycin Treatment

To surface biotinylate Rat 6–derived cultures, cells were detached in 0.04% EDTA in PBS, washed with serum-free DME, and incubated on ice with serum-free DME plus 50 µM sulfo-NHS-LC-biotin (Pierce Chemical Co., Rockford, IL) for 0.5 h. Cells were then washed again and lysed in RIPA buffer, and CDO was immunoprecipitated. Western blotting was then performed as described above, except that HRP-linked streptavidin was used for detection. To assess N-glycosylation of CDO, Rat 6 cells were treated with tunicamycin (5 µg/ml) for 48 h, lysed, and analyzed by Western blotting.

Ectopic Expression of *cdo*

A complete rat *cdo* open-reading frame, including 95 bp of the 5' untranslated region and 633 bp of the 3' untranslated region, was reconstructed from overlapping bacteriophage inserts and ligated into the retroviral expression vector, pBabePuro (Morganstern and Land, 1990). Production of recombinant retroviruses and infection of Rat 6–derived cell lines was performed as previously described (Krauss et al., 1992). Infected cultures were selected in medium that contained 5 µg puromycin per ml, and >1,000 independent, drug-resistant colonies were pooled and analyzed as described in Results.

Chromosomal Localization of Human *cdo*

A portion of the human *cdo* gene was PCR amplified from human monochromosomal somatic cell hybrids. The National Institute of General Medical Sciences human/rodent somatic cell hybrid mapping panel No. 2, version 3 (Coriell Institute for Medical Research, Camden, NJ), was used for chromosomes 1, 6–9, 11, 12, 14, 16, 17, 20, 22, X, and Y. The remaining chromosomes were represented by monochromosomal hybrids: GM11686 (chr. 2); GM11713 (chr. 3); GM11687 (chr. 4); GM11714 (chr. 5); GM11688 (chr. 10); GM11689 (chr. 13); GM11715 (chr. 15); GM12082

(chr. 18); GM10612 (chr. 19); and GM08854 (chr. 21) (Coriell Institute for Medical Research). PCR amplifications were performed with the following primers: 5'-CACAAACAGGCTGGGAGCGA-3' (sense), and 5'-GGGGAAGAAAACATTTAAG-3' (antisense). The identity of the chromosome 11 monochromosomal hybrid was confirmed by PCR of the previously mapped locus catalase (11p13) (Theune et al., 1991). Human *cdo* was further localized with a chromosome 11 regional mapping panel (Coriell Institute for Medical Research).

Results

Isolation, Sequence Analysis, and Tissue Distribution of *cdo*

The R-1a and ER-1-2 mutant rat embryo fibroblast cell lines are resistant to induction of anchorage-independent growth by various oncogenes, and this property is dominant in somatic cell hybridizations (Krauss et al., 1992). Because this dominant resistance could have arisen by the stable expression (or overexpression) of a transformation suppressor gene, a cDNA library constructed from R-1a cells was screened by differential hybridization to isolate genes whose expression was enhanced in R-1a cells, relative to a transformation-sensitive control cell line, PKC3-F4. One cDNA clone isolated in this screen, later named *cdo*, hybridized to an ~8.5-kb mRNA species that reproducibly showed a mild (1.5–2-fold) up-regulation in both R-1a and ER-1-2 cells, relative to the control PKC3-F4 cells (Fig. 1). Interestingly, *cdo* mRNA levels were dramatically down-regulated in PKC3-F4 cells that had been infected with a v-H-*ras*-expressing retrovirus. This down-regulation was incomplete in similarly infected cultures of the *ras*-resistant R-1a and ER-1-2 cell lines (Fig. 1).

Sequence analysis of overlapping cDNAs revealed *cdo* to be a member of the Ig/FNIII repeat family. Fig. 2 A shows the predicted amino acid sequence of rat *cdo*; also shown is the amino acid sequence of a closely related human gene (see below). The rat *cdo* mRNA is predicted to encode a 1,242-amino acid protein, comprised of a 24-amino acid hydrophobic signal sequence followed by an extracellular region of five Ig V-like repeats (Vaughn and Bjorkman, 1996) and three FNIII-like repeats, a 25-residue, membrane-spanning region, and a putative intracellular region of 256 amino acids. A hydrophathy plot (not shown) is consistent with a single membrane-spanning region, suggesting that *cdo* encodes a transmembrane protein with a structure similar to members of the N-CAM family. A schematic representation of the predicted CDO structure is shown in Fig. 2 B. While there are no other IgSF members with this numerical combination of Ig- and FNIII-like repeats in the current databases, individual Ig and FNIII repeats in *cdo* appeared to be most closely related to such

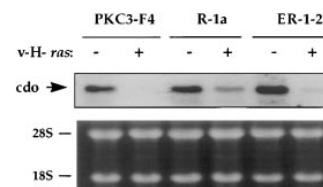
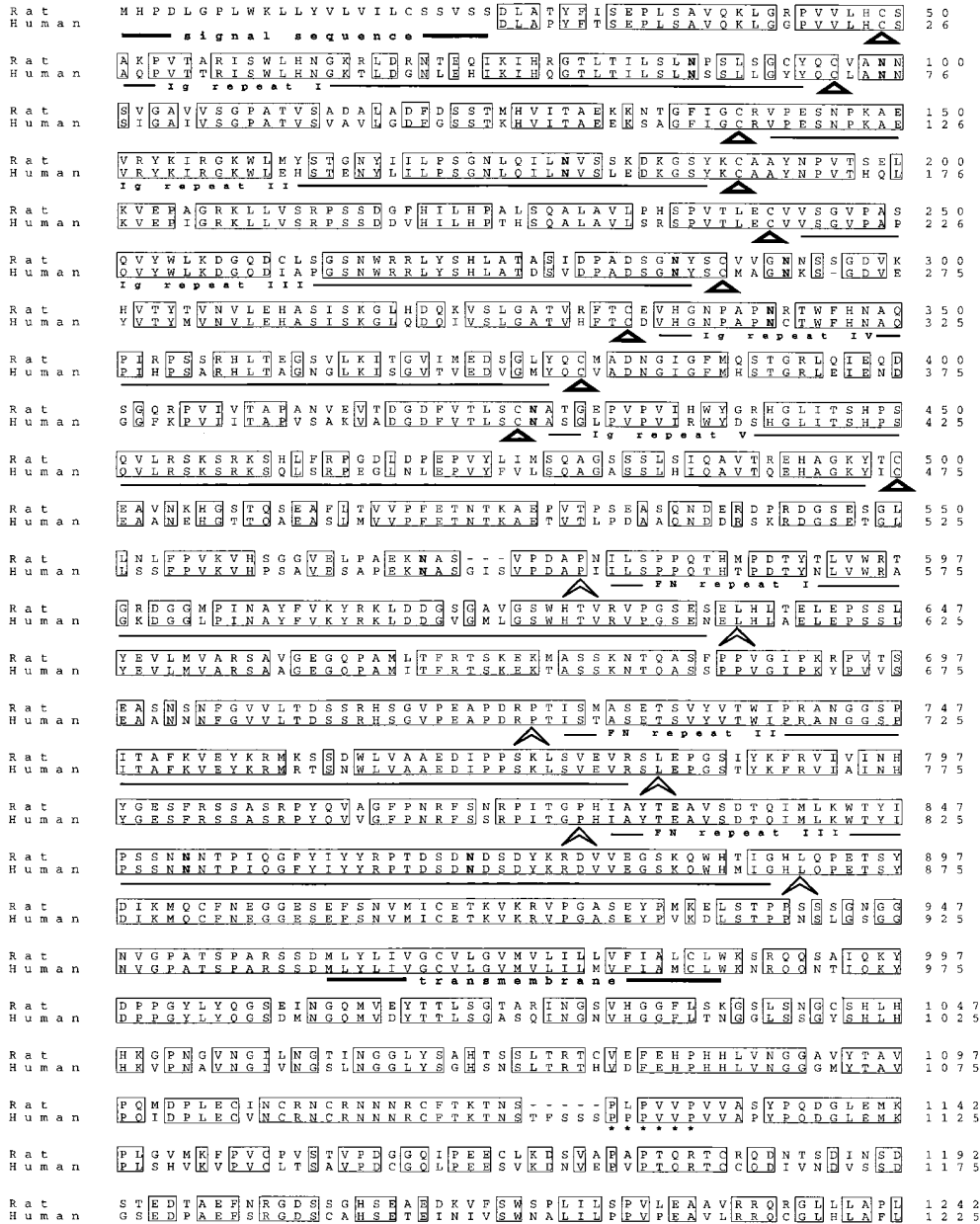


Figure 1. Northern blot analysis of *cdo* mRNA expression in the PKC3-F4, R-1a, and ER-1-2 cell lines, plus or minus infection with a v-H-*ras*-expressing retrovirus. + and -, expressing or not expressing v-H-*ras*, respectively. The ethidium bromide-stained gel (displaying the 28S and 18S ribosomal RNA bands), is shown as a loading control.

A



B

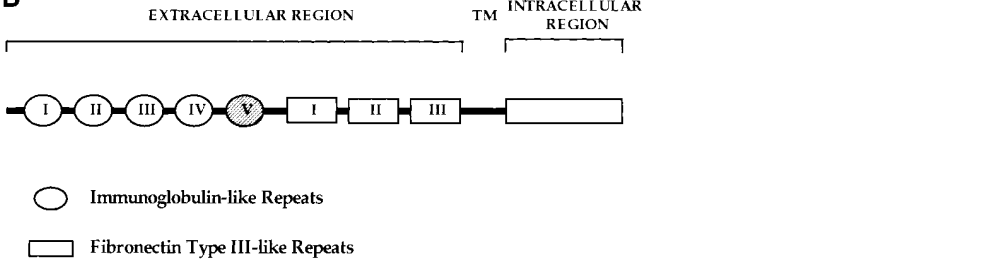


Figure 2. (A) Predicted amino acid sequence of rat and human CDO. Identical residues are boxed. The five Ig-like repeats are indicated by the triangles, which correspond to the two conserved cysteines present in each domain (Williams and Barclay, 1988). The three FNIII-like repeats are indicated by the arrowheads, which correspond to the conserved prolines and leucines at the end of each domain (Patthy, 1990). The putative signal sequence and transmembrane regions are underlined in bold. 10 asparagines in the extracellular region that represent potential sites of N-linked glycosylation are in bold print. A proline-rich stretch in the intracellular region is underscored with asterisks. These sequence data are available from EMBL/GenBank/DBJ, under accession numbers AF004840 (rat), and AF004841 (human). (B) Schematic representation of CDO structure, which contains five Ig-like repeats, three FNIII-like repeats, a transmembrane region (TM), and a 256-amino acid intracellular region. Ig repeat 5 (cross-hatched) is alternatively spliced.

repeats found in rat NB-2 and NB-3 (Ogawa et al., 1996), members of the F11/contactin/F3 subfamily of CAMs (up to 50% amino acid similarity; data not shown). Interestingly, however, the putative intracellular region of *cdo* showed no significant resemblance to any gene currently in the standard databases. A proline-rich stretch in the

intracellular region that might serve as a potential SH3 domain binding site is indicated in Fig. 2 A. 10 potential N-linked glycosylation sites present in the extracellular region are also indicated.
Sequencing of multiple, independent cDNAs revealed a potential for alternative splicing of the fifth Ig repeat,

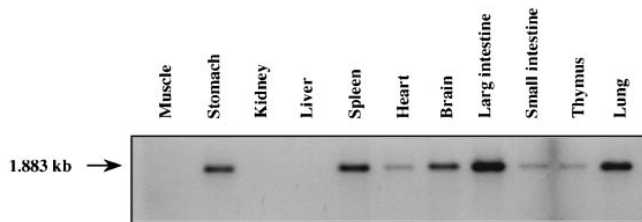


Figure 3. Expression of *cdo* in adult rat tissues. PCR-based “exon-connection” assays for detection of *cdo* transcripts were performed as described in the text.

leading to removal of amino acids 400–516. Reverse transcriptase–PCR analysis of total Rat 6 cell RNA with primers that flank this region produced a major product corresponding to mRNA that encodes the fifth Ig domain, and a minor product corresponding to mRNA that lacks this domain (data not shown). Consistent with the notion that this is a minor splice variant in Rat 6 cells, only one major form of *cdo* mRNA and protein was detected by Northern and Western blot analyses of these cells (see below).

Expression of *cdo* in adult rat tissues was analyzed initially by Northern blotting of poly(A)⁺ RNA derived from brain, liver, kidney, heart, large and small intestines, spleen, thymus, lung, stomach, and skeletal muscle, but only a very faint signal could be detected. This result was reminiscent of that observed for the related gene, *DCC*. We chose, therefore, to use the “exon-connection” strategy originally used by Fearon et al. (1990) to analyze *DCC* expression. A product of the predicted size was found in all of the tissues examined except liver, kidney, and skeletal muscle (Fig. 3). Thus, *cdo* is expressed at low levels in most adult tissues.

Regulation of *cdo* mRNA by Oncogenes and Serum

The PKC3-F4, R-1a, and ER-1-2 cell lines all share the same original parental cell line, Rat 6. Expression of *cdo* mRNA in Rat 6 cells, as well as in Rat 6 cells transformed by different oncogenes, was therefore assessed. Transformation of Rat 6 cells by the *H-ras*, *neu*, *v-src*, *v-raf*, protein kinase C ϵ , or *v-fos* oncogenes led to a dramatic loss of expression of *cdo* (Fig. 4 A). Each of these oncogene-expressing cell lines was grossly morphologically transformed and formed large colonies in soft agar (Borner et al., 1992). In contrast, overexpression of protein kinase C β 1, which led to enhanced mitogen-induced signaling but was a very weak transforming gene (Housey et al., 1988; Borner et al., 1995), had little effect on *cdo* expression. Furthermore, hybridization of a rat *cdo* cDNA probe to Northern blots of RNA from the murine embryo fibroblast line, NIH 3T3, revealed an mRNA species that was indistinguishable in size from that seen in Rat 6 cells, and this mRNA was nearly undetectable in NIH 3T3 cells transformed by a *K-ras* oncogene (Fig. 4 B). Thus, down-regulation of *cdo* mRNA expression correlated with establishment of the transformed phenotype by several different oncogenes in Rat 6 cells and by *ras* oncogenes in two different cell lines.

The possibility that *cdo* expression might be down-regulated by mitogen treatment was examined next. Fig. 4 C demonstrates that when confluent, serum-starved Rat 6

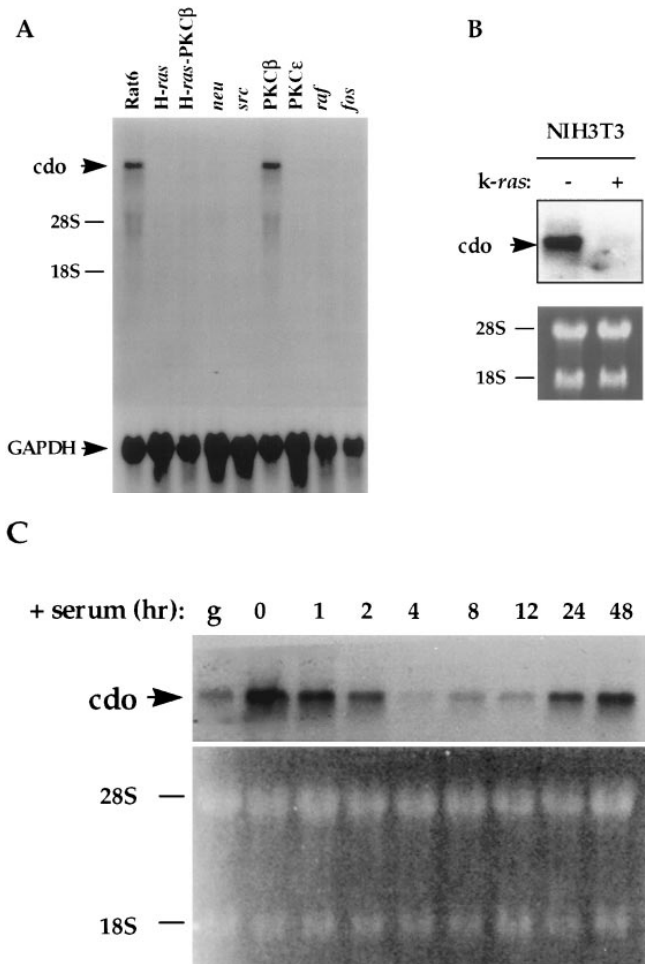


Figure 4. Effect of oncogenes and serum on *cdo* mRNA expression. (A) Northern blot analysis of *cdo* expression in Rat 6 cells and various oncogene-transformed Rat 6 cell derivatives. The lane designations refer to the oncogene expressed in the given Rat 6 cell derivative. The same blot was rehybridized with a rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probe as a loading control. (B) Northern blot analysis of *cdo* expression in NIH 3T3 cells, and NIH 3T3 cells transformed by the *k-ras* oncogene. + and –, expressing or not expressing *k-ras*, respectively. The ethidium bromide–stained gel, displaying the 28S and 18S ribosomal RNA bands, is shown as a loading control. (C) Northern blot analysis of the time course of *cdo* mRNA expression in serum-stimulated Rat 6 cells. Confluent cultures of wild-type Rat 6 cells were serum starved (0 h) and then fed with fresh, serum-containing medium. Lane designations represent the time, in hours, after serum stimulation; lane *g* represents cells growing logarithmically in serum-containing medium. The ethidium bromide–stained gel, displaying the 28S and 18S ribosomal RNA bands, is shown as a loading control.

cells were stimulated by refeeding with fresh, serum-containing medium, *cdo* expression was nearly extinguished within 4 h. Expression began to return within 8–12 h, and by 24–48 h, it had been restored to the level of the original cultures. Parallel cultures that were analyzed for cell cycle progression by flow cytometry indicated that >70% of the cells were in S phase 16 h after serum stimulation, and that the cultures were fully quiescent again 24–48 h after stimulation (data not shown). Thus, steady-state levels of *cdo* mRNA were transiently down-regulated as G₀-arrested

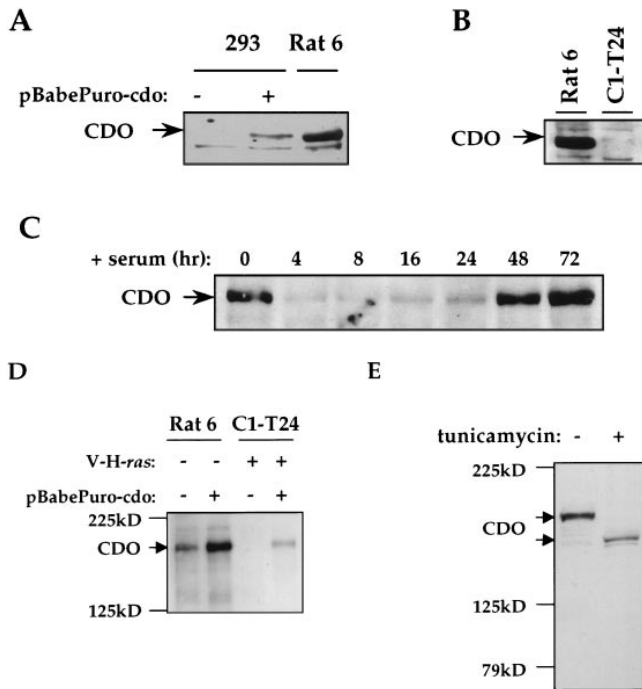


Figure 5. Western blot analyses of CDO protein expression with antisera against the CDO intracellular region. (A) Transient transfection of the pBabePuro/*cdo* vector into 293 cells. + and -, cells were transfected or mock transfected with the expression vector, respectively. (B) Ras-mediated down-regulation of CDO protein levels in Rat 6 cells. C1-T24 cells are a *ras*-transformed Rat 6 cell subclone. (C) Serum-mediated down-regulation of CDO protein levels in Rat 6 cells. Confluent cultures of wild-type Rat 6 cells were serum starved (0 h) and then fed with fresh, serum-containing medium. Lane designations represent the time, in hours, after serum stimulation; note that the times after stimulation at which CDO protein was analyzed are not identical to those used in analysis of *cdo* mRNA in Fig. 2. (D) Cell surface expression of CDO. Rat 6 and C1-T24 cell derivatives were surface biotinylated and detected on Western blots with HRP-linked streptavidin. + and -, cells did, or did not, stably express the pBabePuro/*cdo* vector. (E) N-linked glycosylation of CDO. + and -, Rat 6 cells treated or not treated with tunicamycin, respectively, and then analyzed by immunoblotting with CDO antisera.

cells reentered the cell cycle and were restored as the cultures regained quiescence. Fig. 4 C also shows that logarithmically growing cells had lower levels of *cdo* mRNA than did confluent, serum-starved cells. This appeared not to be due simply to cell proliferation, per se, or solely to the fact that these growing cultures were in the presence of serum, because growth arrest of such cultures by removal of serum did not significantly increase *cdo* expression (data not shown). These data indicate that cell density and/or cell-cell contact might also play a role in *cdo* mRNA regulation.

Analysis of CDO Protein in Rat 6 Cells

A complete *cdo* open reading frame was inserted into the retroviral expression vector, pBabePuro. This vector was transiently transfected into the human embryonic kidney cell line, 293, and Western blot analysis performed on total cell lysates with polyclonal rabbit antisera raised against

the intracellular region of *cdo*. A protein of ~190 kD was detected in transfected cells, but not in mock-transfected controls (Fig. 5 A). An immunoreactive protein with similar mobility was detected in Rat 6 cell lysates (Fig. 5 A). A nonspecific, cross-reactive band of lower molecular weight is also visible in mock-transfected 293 cells and, frequently, in Rat 6 cells. Consistent with the RNA analyses described above, CDO protein levels were dramatically down-regulated in *ras*-transformed Rat 6 cells (C1-T24 cells; Fig. 5 B). Likewise, serum stimulation of quiescent Rat 6 cultures led to transient down-regulation of CDO protein levels (Fig. 5 C). It was not possible to perform similar studies with NIH 3T3 cells, because the antibodies to CDO appear to be specific for rat.

The amino acid sequence of *cdo* predicts a transmembrane protein. To confirm that CDO was expressed on the cell surface, various Rat 6 cell derivatives were surface biotinylated. CDO was then immunoprecipitated and detected on Western blots with HRP-coupled streptavidin. As shown in Fig. 5 D, a band of the appropriate molecular weight was observed in Rat 6 cells, but was not present in *ras*-transformed C1-T24 cells. Furthermore, the signal intensity of the band was increased in Rat 6 cells that overproduce CDO, and the band was restored in C1-T24 cells that ectopically produce this protein (see below for a description of these cell lines). It is concluded that CDO is expressed on the cell surface.

Members of the IgSF are typically modified in their extracellular region by N-linked glycosylation (Brummendorf and Rathjen, 1995). To test whether CDO is also N-glycosylated, Rat 6 cells were treated with tunicamycin, an inhibitor of this process. CDO from treated cells displayed a significant decrease in apparent molecular weight (Fig. 5 E), consistent with the notion that it is a glycoprotein.

Effect of Cell-Substratum Adhesion on *cdo* RNA and Protein Expression

The expression pattern of *cdo* in Rat 6 and NIH 3T3 cells (Fig. 4) suggests that *cdo* might encode a protein with growth inhibitory or transformation suppressor function. Because the primary defect in the R-1a and ER-1-2 mutant cell lines is an inability to proliferate in an anchorage-independent manner in response to *ras* (Krauss et al., 1992; Kang and Krauss, 1996), it was of interest to determine the effects of cell-substratum adhesion on the expression of *cdo*. Rat 6 cells suspended in methylcellulose-containing medium had significantly lower steady state levels of *cdo* mRNA than did parallel adherent cultures. A similar effect was observed in NIH 3T3 cells (Fig. 6). *cdo* mRNA levels were consistently lower in suspension cultures than in adherent cultures of low cell density (data not shown). It seems likely, however, that the decrease in steady state *cdo* mRNA levels seen in the methylcellulose cultures of Rat 6 cells may reflect a combination of two phenomena: (a) loss of cell anchorage; and (b) the low cell density typically used for these suspension cultures, since decreased cell density in adherent cultures also led to partially reduced levels of *cdo* mRNA (Fig. 4 C, and see above).

Although *cdo* mRNA levels were down-regulated in non-adherent Rat 6 cells, some level of expression was always

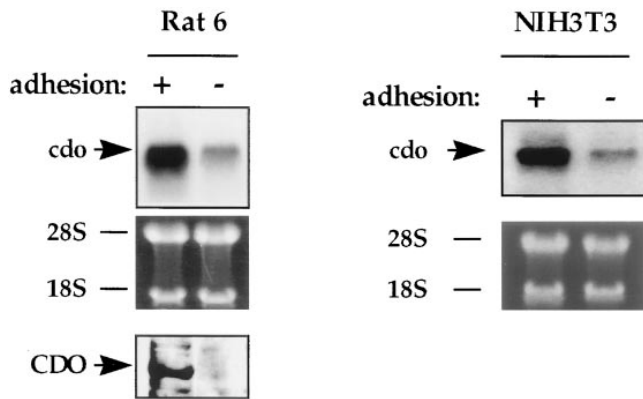


Figure 6. Regulation of *cdo* mRNA and protein by cell–substratum adhesion in Rat 6 and NIH 3T3 cells. + and –, cells were cultured on plastic dishes or suspended in methylcellulose-containing medium, respectively. CDO protein and *cdo* mRNA were analyzed by Western and Northern blotting techniques, respectively. For the Northern blots, the ethidium bromide–stained gel, displaying the 28S and 18S ribosomal RNA bands, is shown as a loading control.

detectable in these cultures. In contrast, CDO protein could not be detected in suspension cultures (Fig. 6). These data raised the possibility that CDO protein levels might be regulated by cell–substratum adhesion by a mechanism that is independent of *cdo* mRNA expression. Additional experiments that address this point are described below.

Ectopic Expression of *cdo*

To test the hypothesis that cell–substratum adhesion can regulate CDO protein levels independently of *cdo* mRNA expression, we sought to express a *cdo* cDNA from a promoter whose activity was not influenced by cell culture conditions. To achieve this, parental Rat 6 cells and a *ras*-transformed derivative, C1-T24, were infected with a recombinant retrovirus that drives expression of *cdo* from the viral long terminal repeat. The cells were infected with either the *cdo*-expressing virus or, as a control, a virus that lacked a cDNA insert. Infectants were then pooled and the polyclonal populations were analyzed.

Ectopic expression of *cdo* was assessed by Northern and Western blot analyses of infected cells cultured under both adherent and nonadherent conditions. As shown in Fig. 7 A, Rat 6 and C1-T24 cells that were infected with the *cdo* virus (Rat 6/*cdo* and C1-T24/*cdo* cells, respectively) expressed abundant, vector-derived mRNA whether they were attached to plates or suspended in methylcellulose. When adherent cultures were tested, Rat 6/*cdo* cells produced significantly more immunoreactive CDO protein than control vector-infected cells (Fig. 7 A). C1-T24/*cdo* cells also produced large quantities of CDO protein when grown on tissue culture plates (Fig. 7 A). Control vector-infected C1-T24 cells expressed no detectable CDO, similar to the parental C1-T24 cell line shown in Fig. 5 B (see also Fig. 7 B). Interestingly, no CDO protein was produced when any of these cell lines was cultured under nonadherent conditions, even though they stably expressed exogenous, vector-derived *cdo* mRNA (Fig. 7 A). We have previously

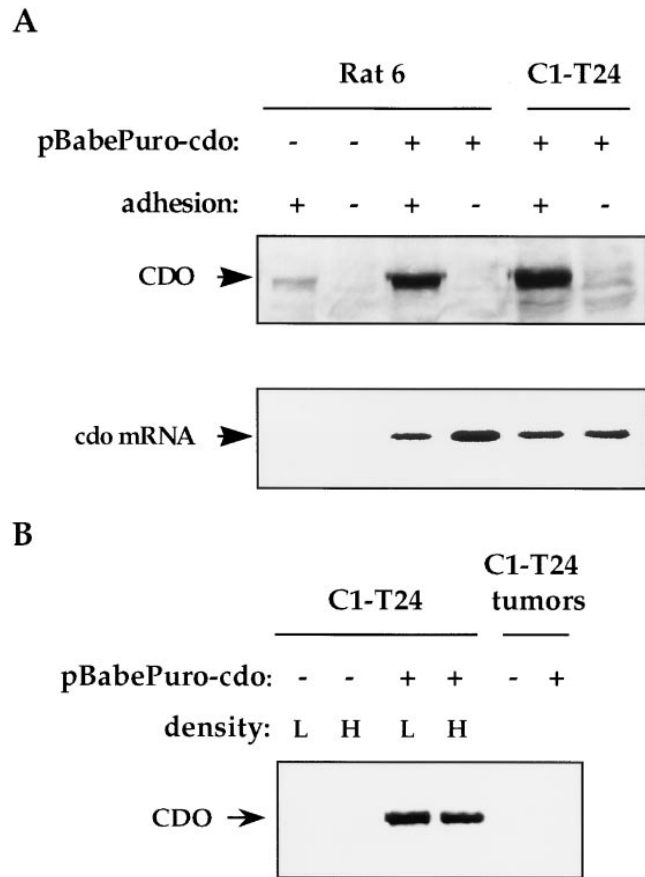


Figure 7. Regulation of ectopic CDO protein production by cell–substratum adhesion. (A) Rat 6 and C1-T24 cells were infected with recombinant pBabePuro/*cdo* virus (+) or pBabePuro virus lacking a cDNA insert (–), and were cultured on plastic dishes (+ adhesion) or suspended in methylcellulose-containing medium (– adhesion). CDO protein expression and mRNA expression were analyzed by Western and Northern blotting techniques, respectively. Prolonged exposure of the Northern blot revealed the endogenous *cdo* mRNA species in the first two lanes, similar to the data presented in Fig. 6. (B) C1-T24 cells were infected with recombinant pBabePuro/*cdo* virus (+) or pBabePuro virus lacking a cDNA insert (–), and were then cultured on plastic dishes at low (L) or high (H) cell density. Also shown are extracts from tumors derived from C1-T24/*puro* (–) and C1-T24/*cdo* (+) cells. Samples were analyzed for CDO protein expression by Western blotting techniques. Western blots were also stained with Ponceau S to confirm that protein samples were not degraded.

demonstrated that production of several cell cycle proteins is not altered by culturing these cells in methylcellulose-containing medium (Kang and Krauss, 1996). Furthermore, immunoblotting of these same cell extracts with antibodies to a different cell surface protein, the insulin-like growth factor I receptor, did not reveal significant differences between adherent and nonadherent cultures in the expression of this protein (data not shown). Loss of CDO production in nonadherent cultures therefore was not due to a nonspecific effect, nor was it due to undetected damage to the cells. These data are consistent with the data described in Fig. 5, and indicate a requirement, at least in Rat 6 cells, for cell–substratum adhesion for stable production of CDO protein.

The failure of Rat 6 cells to produce either endogenous or ectopically expressed CDO protein in the absence of cell–substratum adhesion might have arisen, at least in part, as a result of the low cell density in the methylcellulose cultures used for the nonadherent conditions, rather than as an absolute need for cell anchorage. To distinguish between these possibilities, we cultured C1-T24/*cdo* cells at low and high cell densities on tissue culture plates and measured CDO levels by immunoblotting. C1-T24/*cdo* cells are most appropriate for addressing this question because they express only the vector-derived *cdo* mRNA which, unlike the endogenous *cdo* gene, is not regulated by cell density or cell anchorage. As shown in Fig. 7 B, adherent cultures of C1-T24/*cdo* cells harvested at low and high cell densities each produced abundant CDO protein, indicating that loss of cell substratum adhesion was most likely to be responsible for the effects observed in Fig. 7 A.

As would be expected of cells that failed to produce the exogenously derived protein, there was no difference between C1-T24/*cdo* cells and control vector-infected C1-T24 cells in their frequency of colony formation in soft agar (~20% cloning efficiency). The tumorigenicity of C1-T24/*cdo* and control vector-infected C1-T24 cells was also tested. When 10⁶ cells of each cell line were injected into the flanks of nude mice, both produced tumors of 1-cm diam in 10 d. The tumors were then excised, and tested for expression of CDO protein. Similar to what was observed with the nonadherent cultures, tumors derived from C1-T24/*cdo* cells produced barely detectable levels of CDO (Fig. 7 B), further indicating a need for engagement of the cells with an appropriate substratum for stable production of this protein.

Finally, ectopic expression of CDO protein in adherent cultures of Rat 6/*cdo* and C1-T24/*cdo* cells did not alter the gross morphology or growth properties of either cell type when compared with their respective vector control or parental cell lines (data not shown). Notably, no increase in cell–cell aggregation was observed, as might be expected for ectopic expression of a cell surface molecule that mediated homophilic cell adhesion.

It should be noted that the *ras*-resistant mutant cells also did not produce CDO protein when cultured in suspension (data not shown). Thus, the failure of the mutant cell lines to down-regulate *cdo* mRNA levels to the same extent observed in transformation-sensitive control cells presumably does not account for their resistance to anchorage-independent growth. These data do not, however, exclude a role for CDO as a transformation suppressor in other systems where stable expression of the protein may occur.

Sequence and Chromosomal Localization of a Human Gene Closely Related to *cdo*

A Southern “zoo” blot containing DNA from various species revealed that mouse and human DNA displayed strong cross-species hybridization to a rat *cdo* cDNA probe, while chicken, toad (*Xenopus laevis*), and zebrafish DNA displayed a clearly detectable, but weaker signal (data not shown). Screening of human fetal brain and fetal lung libraries yielded clones that corresponded to a single closely related gene. These human cDNAs hybridized to an mRNA species present in several human neuroblastoma cell lines

that was indistinguishable in size from *cdo* mRNA in Rat 6 cells (data not shown).

The predicted amino acid sequence of this human gene is shown in comparison to that of rat *cdo* in Fig. 2 A. The human and rat genes have identical domain structures in their extracellular regions, and their intracellular regions, which do not resemble other known proteins, are also highly related. Across their full lengths (except for the signal sequence which was missing from the human cDNA clones obtained), the two proteins are ~81% identical and ~96% similar. The relative positions of amino acids that define the Ig- and FNIII-like domain consensus sequences are conserved between the two predicted proteins, as are all 10 potential N-linked glycosylation sites. Two potential microexons are apparent in the human sequence: one is a three–amino acid insertion between Ig-like repeat 5 and FNIII-like repeat 1; the second is a five–amino acid insertion that is immediately adjacent to the proline-rich sequence found in the cytoplasmic tail. Although we cannot rule out the existence of human genes that are even more closely related to rat *cdo*, in this article, this gene will be referred to as human *cdo*.

To identify a chromosomal location for human *cdo*, a panel of human/rodent monochromosomal hybrids was screened by PCR with primers that amplify human, but not rodent, *cdo* DNA. These studies mapped human *cdo* to chromosome 11 (data not shown). PCR analysis of a chromosome 11 regional mapping panel showed that *cdo* mapped to the interval 11q23–24 as defined by the break-

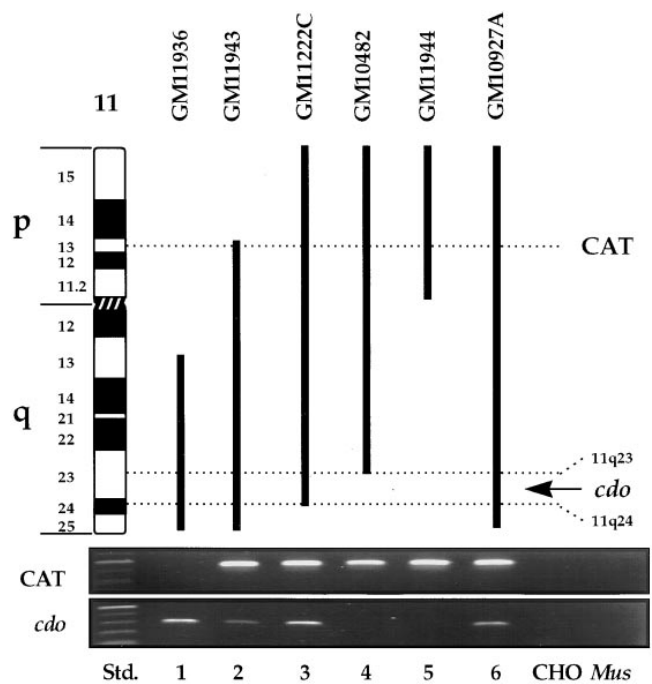


Figure 8. Regional localization of human *cdo* to chromosome 11q23–24. PCR amplification of human *cdo* and, as a control, the catalase locus (*CAT*; *11p13*) in a regional mapping panel containing the displayed portions of human chromosome 11 in a rodent cell background. Ethidium bromide–stained gels are shown. *CHO*, genomic DNA from CHO cells; *Mus*, genomic DNA from mouse.

points of the hybrids GM10482 (11q23) and GM11222C (11q24). Hybrid GM11222C was positive for *cdo*, whereas GM10482 was negative (Fig. 8). This region has substantial overlap with similarly sized regions that display LOH in a high percentage of human breast, ovarian, and lung cancers (Negrini et al., 1995; Rasio et al., 1995; Gabra et al., 1996). PCR for the catalase locus (11p13) was used as a control to confirm the identity of the hybrids (Fig. 8).

Discussion

Proteins of the Ig/FNIII family play important roles in a variety of developmental and physiological processes. Additionally, expression of Ig/FNIII proteins can be highly dynamic and cell type specific, although little is known of the signaling pathways that regulate such expression (Edelman and Crossin, 1991; Brummendorf and Rathjen, 1995; Cunningham, 1995). We report here the molecular cloning and characterization of rat CDO, a novel cell surface glycoprotein of the Ig/FNIII family. A *cdo* cDNA was isolated by screening a genetically dominant, oncogene-resistant mutant cell line for genes whose expression was enhanced relative to a transformation-sensitive control cell line. *cdo* mRNA and protein were dramatically down-regulated in rat and mouse embryo fibroblast cell lines that were transformed by various oncogenes. Additionally, *cdo* mRNA and protein levels were strongly, but transiently, reduced when confluent, quiescent cells were stimulated to reenter the cell cycle. These observations raise the possibility that CDO may serve as a negative regulator of cell proliferation, transformation, and/or tumorigenesis. Consistent with this possibility, a human gene that is very closely related to rat *cdo* mapped to chromosome 11q23–24, a region that exhibits LOH in a high percentage of human breast, ovarian, and lung cancers (Negrini et al., 1995; Rasio et al., 1995; Gabra et al., 1996). *cdo* therefore joins a growing list of genes that are not only down-regulated at the mRNA level in transformed cell lines or tumors, but may also have transformation suppressor function. Such genes include those that encode components of the extracellular matrix (fibronectin, collagen); cell surface proteins (E- and H-cadherin, connexins 26 and 43, certain integrins, and maspin); and proteins that organize the actin cytoskeleton (vinculin, α -actinin, tropomyosin 1) (Plantefaber and Hynes, 1989; Giancotti and Ruoslahti, 1990; Frixen et al., 1991; Vleminckx et al., 1991; Fernandez et al., 1992; Gluck et al., 1993; Prasad et al., 1993; Akamatsu et al., 1996; Hirsch et al., 1996; Lee, 1996; Sheng et al., 1996; Travers et al., 1996).

In addition to regulation of *cdo* mRNA levels by oncogenes and serum, CDO protein levels were powerfully controlled by cell–substratum adhesion, via a mechanism that was independent of *cdo* mRNA expression. Rat 6 cells and the Ras-transformed derivative cell line C1-T24 each displayed a cell anchorage requirement for stable production of CDO, even when this protein was derived from an ectopically expressed mRNA, whose levels were not altered by the culture conditions. Preliminary experiments indicate that when suspended C1-T24/*cdo* cells were allowed to attach and spread on fibronectin-coated dishes, CDO was restored to maximal levels 2 h after replating. Furthermore, attachment-mediated production of CDO

was partially blocked by cytochalasin D (Kang, J.-S., and R.S. Krauss, unpublished observations). These data suggest a possible role for integrin-mediated signaling and cytoskeletal organization in this process. They also raise the possibility that stable production of CDO may require an appropriate extracellular matrix microenvironment. Such a requirement would have important implications for cell type specificity in the production of CDO and other proteins that might be regulated in a similar fashion. It will be very interesting to determine whether anchorage or specific extracellular matrix proteins are required for stable production of other Ig/FNIII family members. It is not known whether the loss of CDO protein in suspension cultures occurred at the level of translation or stability. Zhu et al. (1996) have reported that translation of cyclin D1 mRNA is dependent on cell–substratum adhesion, but unlike the observations described here for CDO, this effect of cell anchorage was lost when ectopic cyclin D1 transcripts lacked untranslated regions.

Most CAMs of the IgSF can mediate homophilic binding, although DCC may be an exception (Fearon, 1996). However, Rat 6 and C1-T24 cells that ectopically expressed CDO did not exhibit enhanced cell–cell aggregation. Furthermore, preliminary studies indicate that a soluble fusion protein that contains the entire CDO extracellular region is unable to bind to Rat 6 or Rat 6/*cdo* cells, each of which has abundant CDO on their cell surface (Kang, J.-S., and R.S. Krauss, unpublished observations). These observations indicate that Rat 6 cells do not express a ligand for CDO on their cell surface, and also that CDO is probably not a homophilic CAM. Therefore, it will also be interesting to determine the effects of cell anchorage on CDO production in a cell system in which a known ligand for CDO is present. The presence of a ligand could conceivably stabilize or stimulate production of CDO, even in the absence of optimal cell anchorage. The levels of CDO at the cell surface may therefore be governed by complex interactions between (a) signals that can regulate *cdo* gene expression, such as mitogenic stimuli; and (b) signals that can regulate CDO protein production, such as appropriate cell–substratum interactions and, possibly, the presence of ligands.

Members of the Ig/FNIII repeat family can be organized into subfamilies by the number of Ig- and FNIII-like repeats they contain in their extracellular regions (Brummendorf and Rathjen, 1995; Cunningham, 1995). We are not aware of any previously reported members of this family that have the five Ig plus three FNIII combination of repeats. CDO's unique intracellular region also distinguishes it from other Ig/FNIII proteins. *cdo* is therefore a founding member of a new subfamily within the Ig/FNIII repeat family. Given the structural resemblance of CDO to N-CAM, DCC, and other Ig/FNIII family members, it is tempting to speculate that CDO might also play a role in cell migratory events during development of the nervous system and/or other organ systems. Consistent with this notion, *cdo* mRNA is expressed at high levels in several human neuroblastoma cell lines (Gao, M., and R.S. Krauss, unpublished observations); in addition, preliminary in situ hybridization studies indicate that *cdo* mRNA is expressed at high levels in particular regions of the developing rat spinal cord and, interestingly, limb bud (Brose, K., J.-S.

Kang, R.S. Krauss, and M. Tessier-Lavigne, unpublished studies). Because under some conditions Rat 6 cells express *cdo* mRNA but not its protein product, it will be important to identify cell surface production of CDO protein by particular cell types during development to understand its function. Understanding CDO's possible functions in development and oncogenesis will also require analysis of the protein's long cytoplasmic tail, which bears no significant resemblance to other known proteins. The dramatic regulation of *cdo* mRNA and protein production by mitogenic and anchorage-related stimuli, combined with CDO's unique intracellular region, indicate that further studies of this protein should yield interesting new insights into cell proliferation and/or motility, and how such processes may go awry during tumorigenesis. Such studies are in progress.

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