The EGF-CFC family: novel epidermal growth factor-related proteins in development and cancer

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Abstract

The EGF-CFC gene family encodes a group of structurally related proteins that serve as important competence factors during early embryogenesis in Xenopus, zebrafish, mice and humans. This multigene family consists of Xenopus FRL-1, zebrafish one-eyed-pinhead (oep), mouse cripto (Cr-1) and cryptic, and human cripto (CR-1) and criptin. FRL-1, oep and mouse cripto are essential for the formation of mesoderm and endoderm and for correct establishment of the anterior/posterior axis. In addition, oep and cryptic are important for the establishment of left-right (L/R) asymmetry. In zebrafish, there is strong genetic evidence that oep functions as an obligatory co-factor for the correct signaling of a transforming growth factor-β (TGFβ)-related gene, nodal, during gastrulation and during L/R asymmetry development. Expression of Cr-1 and cryptic is extinguished in the embryo after day 8 of gestation except for the developing heart where Cr-1 expression is necessary for myocardial development. In the mouse, cryptic is not expressed in adult tissues whereas Cr-1 is expressed at a low level in several different tissues including the mammary gland. In the mammary gland, expression of Cr-1 in the ductal epithelial cells increases during pregnancy and lactation and immunoreactive and biologically active Cr-1 protein can be detected in human milk. Overexpression of Cr-1 in mouse mammary epithelial cells can facilitate their in vitro transformation and in vivo these Cr-1–transduced cells produce ductal hyperplasias in the mammary gland. Recombinant mouse or human cripto can enhance cell motility and branching morphogenesis in mammary epithelial cells and in some human tumor cells. These effects are accompanied by an epithelial-mesenchymal transition which is associated with a decrease in β-catenin function and an increase in vimentin expression. Expression of cripto is increased several-fold in human colon, gastric, pancreatic and lung carcinomas and in a variety of different types of mouse and human breast carcinomas. More importantly, this increase can first be detected in premalignant lesions in some of these tissues. Although a specific receptor for the EGF-CFC proteins has not yet been identified, oep depends upon an activin-type RIIB and RIB receptor system that functions through Smad-2. Mouse and human cripto have been shown to activate a ras/raf/MAP kinase signaling pathway in mammary epithelial cells. Activation of phosphatidylinositol 3-kinase and Akt are also important for the ability of CR-1 to stimulate cell migration and to block lactogenic hormone-induced expression of β-casein and whey acidic protein. In mammary epithelial cells, part of these responses may depend on the ability of CR-1 to transactivate erb B-4 and/or fibroblast growth factor receptor 1 through an src-like tyrosine kinase.
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The EGF-CFC gene family

A new family of genes, the EGF-CFC family, that encodes extracellular growth factor-like and/or co-receptor-like proteins has been identified in the human, mouse, frog and zebrafish. Members of this family include human cripto-1 (CR-1) (also known as teratocarcina-derived growth factor-1 (TDGF-1)) and criptin (unpublished Human Genome Sciences Inc. patent number S5981215), mouse cripto-1 (Cr-1/tdgf-1) and cryptic, Xenopus FRL-1 and zebrafish one-eyed pinhead (oep) (Table 1) (Ciccodicola et al. 1989, Dono et al. 1993, Kinoshita et al. 1995, Shen et al. 1997, Zhang et al. 1998). CR-1 was serendipitously isolated in a screen for glucose-6–phosphate dehydrogenase as a chimeric cDNA from a human NTERA2/D1 embryonal carcinoma cDNA library (Ciccodicola et al. 1989). The mouse gene, Cr-1, was subsequently identified and cloned from a mouse embryo cDNA library (Dono et al. 1993). The closely related mouse cryptic gene was cloned by differential RT-PCR from a mouse embryonic body-derived mesoderm cDNA library, and criptin from a human pancreatic carcinoma cDNA library (Shen et al. 1997). Similarly, Xenopus laevis FRL-1 was isolated in a functional screening in yeast with the intent of identifying fibroblast growth factor receptor-1 (FGFR-1) activating genes from a Xenopus embryonic mesoderm cDNA library (Kinoshita et al. 1995). More recently, the zebrafish oep gene has been positionally cloned and identified (Zhang et al. 1998).

Genomic organization of mouse and human cripto

CR-1 maps centromerically to a region on chromosome 3p21.3 that is adjacent to or possibly part of a region which is frequently deleted or exhibits loss of heterozygosity (LOH) in a subpopulation of head and neck, renal, gastric, bladder, breast and lung carcinomas (Dono et al. 1991, Saccone et al. 1995, Todd et al. 1996, Sekido et al. 1998, Cuthbert et al. 1999). Interestingly, the β-catenin gene (CTNNB1) is located at the chromosomal region 3p21.3–p22 while the activin receptor IIB is located at 3p22 (Kraus et al. 1994, Bondestam et al. 1999). These two genes may be involved in intracellular signaling by EGF-CFC proteins (see below). The mouse and human cripto genes consist of six exons and five introns and possess inverted Alu and B1 sequence elements respectively, and AUUU(A)-type Kamen-like sequences in a large 3' untranslated region, suggesting that they encode relatively short-lived mRNA species (Fig. 1) (Dono et al. 1991, 1993). The human CR-1 coding sequence containing the six exons is 4.8 kb in length. There is an excellent conservation of the exon-intron structure in the region of exon 4 which contains the EGF-like motif, while exons 1 and 3 of the mouse are 15 and 33 nucleotides shorter respectively than the corresponding human exons (Dono et al. 1991, 1993). The 5' upstream genomic sequences of mouse Cr-1 and human CR-1 between -610 and -1 from the most distal translation start sites are quite dissimilar (Baldassarre et al. 2000). Several TATA and CAAT boxes are present in the mouse Cr-1 promoter region while in the human CR-1 promoter these sequences are missing. These data suggest that regulation of expression of the mouse and human cripto genes may not be entirely similar. Multiple copies of cripto-specific sequences are present in the human and mouse genomes (Dono et al. 1991, Saccone et al. 1995, Liguori et al. 1996, 1997, Scognamiglio et al. 1999). At least five other human CR-1–related pseudogenes and two mouse Cr-1 pseudogenes have been identified (Scognamiglio et al. 1999). The CR-2, CR-4 and CR-5 genes are truncated at the 5'-end and have accumulated point mutations, deletions and insertions (Scognamiglio et al. 1999). These genes map to chromosomes 2q37, 6p25 and 3q22 respectively while CR-6 maps to 19q13.1. The CR-3 pseudogene which maps to the Xq21–q22 region and the mouse Cr-2 pseudogene are intronless genes that have many characteristics of a retroposon but nevertheless have the potential to code for functional proteins that differ from the proteins encoded by either CR-1 or Cr-1 by only five amino acids.

CR-1 and Cr-1 encode major mRNA species of approximately 2.2 kb. In some cases, less abundant transcripts of about 1.7, 3.0, 3.2 and 3.5 kb have been detected in midgestation mouse embryos and in primary and metastatic human colon and hepatic carcinomas, suggesting that they may arise by the use of different polyadenylation sequences, by alternative splicing or by the use of an alternative initiation site for transcription (Ciccodicola et al. 1989, Dono et al. 1993, Johnson et al. 1994, Baldassarre et al. 2000). In this regard, a truncated CR-1 protein of 145 amino acids may be expressed from the 1.7 kb mRNA transcript in metastatic human

Table 1 EGF-CFC family and their function.

<table>
<thead>
<tr>
<th>EGF-CFC proteins</th>
<th>Function</th>
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<tbody>
<tr>
<td>Human Cripto-1 (CR-1)</td>
<td>Gastrulation/mammary morphogen ?</td>
</tr>
<tr>
<td>Mouse Cripto-1 (Cr-1)</td>
<td>Primitive streak</td>
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<tr>
<td>Mouse Criptin</td>
<td>A/P axial formation and positioning</td>
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<tr>
<td>Mouse Cr-1 (Cr1)</td>
<td>Mesoderm and endoderm formation</td>
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<tr>
<td>Mouse Cryptic</td>
<td>Cardiomyocyte development</td>
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<td>Mouse L/R symmetry</td>
<td>Cardiac defects/right isomerization</td>
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<tr>
<td>Mouse Postnatal lethality</td>
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<tr>
<td>Xenopus FRL-1</td>
<td>Mesoderm formation</td>
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<tr>
<td>Xenopus Neuroectoderm formation</td>
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<tr>
<td>Zebrafish one-eyed pinhead (oep)</td>
<td>A/P axial formation and positioning</td>
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<td>Zebrafish A/P axial formation and positioning</td>
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<tr>
<td>Zebrafish L/R symmetry</td>
<td></td>
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<tr>
<td>Zebrafish Mesoderm and endoderm formation</td>
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<tr>
<td>Zebrafish Ventral neuroectoderm</td>
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Figure 1 (A) Schematic diagram of the human CR-1 gene and mRNA. Hatched areas represent the six exons, with the red exon encoding EGF-like motif. Numbers represent the number of bases in each region (obtained from Baldassarre et al. 2000). (B) Amino acid sequence alignment of cripto-related proteins, zebrafish oep, mouse cripto, mouse cryptic and Xenopus FRL-1. Green and yellow bars indicate amino acid identity among 4/4 and 3/4 proteins respectively. Blue and red underline represent EGF-like motif and cysteine-rich domains respectively (data obtained from Zhang et al. 1998 and reproduced with permission of the author). (C) Sequence alignment of EGF-like motifs. Green and red shaded areas represent amino acid identity in three or more proteins and in six proteins respectively. Conservation of six cysteines noted in yellow with disulfide bonds between cysteines (Cys) 1 and 3, 2 and 4, and 5 and 6 constitute loops A, B and C respectively. Note absence of residues between Cys 1 and 2 in cripto-related proteins compared with mouse EGF.

colorectal carcinomas and in hepatic colon metastases due to the use of a second CUG initiation codon (at leucine 44 in the coding sequence) that eliminates the use of the first two exons and thereby deletes 43 amino acids from the N-terminus of the protein. In the mouse, low levels of Cr-1 mRNA expression can be detected by RNase protection assays in the adult spleen, heart, lung and in distinct regions of the brain (Dono et al. 1993, Johnson et al. 1994). Adult tissues in the human that express low levels of mRNA transcripts for the 188 amino acid isoform of CR-1 as detected by RT-PCR include lung, kidney, brain, testis, ovary and spleen (Baldassarre et al. 2000). Tissues expressing only the truncated form of CR-1 include pancreas, heart, stomach, small intestine, mammary gland, skeletal muscle and liver. Likewise, mouse cryptic contains two in-frame potential translation initiation AUG start sites in which the second AUG start site would code for a protein that is truncated at the NH2-terminus by 13 amino acids (Shen et al. 1997).

Physiochemical properties of the EGF-CFC proteins

The EGF-CFC family of proteins has a general profile consisting of a potential NH2-terminal signal peptide, a
modified epidermal growth factor (EGF)-like motif, a conserved cysteine-rich motif (CFC region) and a short hydrophobic COOH-terminus which in some cases contains additional consensus sequences for potential glycosyl-phosphatidylinositol (GPI) cleavage and attachment (Fig. 1 and Table 2) (Ciccodicola et al. 1989, Dono et al. 1993, Kinoshita et al. 1995, Shen et al. 1997, Zhang et al. 1998, Gritsman et al. 1999). An overall sequence identity of 22–32% exists between mouse and human cripto, mouse cryptic, human criptin, *Xenopus* FRL-1 and zebrafish oep. Within the EGF-like domain of the EGF-CFC proteins, there is nearly a 60–70% sequence similarity, while in the CFC region the similarity ranges from 35 to 48%. The variant EGF-like motif is a region of approximately 40 amino acids containing six cysteine residues that can form three intramolecular disulfide bonds (Fig. 1). This region has the thermodynamic potential to refold into a tertiary structure. While in the canonical EGF motif, three loops (A, B and C) can be recognized, the variant EGF-like motif in the EGF-CFC proteins lacks the A loop, possesses a truncated B loop and has a complete C loop which differentiates the EGF-CFC proteins from other members in the EGF superfamily of peptides (Fig. 2). Members of the EGF superfamily of peptides include EGF, transforming growth factor α (TGFα), amphiregulin (AR), heparin-binding EGF-like growth factor (HB-EGF), betacellulin (BTC), epiregulin (EPI), tomoregulin (TR) and three distinct genes in the neuregulin (NRG) family each coding for at least 15 distinct isoforms of α, β or γ heregulin (HRG) (Uchida et al. 1999, Klapper et al. 2000). The solution structure as determined by nuclear magnetic resonance (NMR) of the EGF-like domains from these EGF-related peptides is very similar since the B loop contains two anti-parallel β-sheets and a β-turn with a hinge region separating this region from the C loop which contains an additional smaller anti-parallel β-sheet and a β-turn. All these peptides bind to and function through homo- and/or heterodimers of the erb B type I tyrosine kinase family of cell surface receptors which includes the epidermal growth factor receptor (EGFR/c-erb B), c-erb B-2/HER-2, c-erb B-3/HER-3 and c-erb B-4/HER-4 (Klapper et al. 2000). Conserved amino acids within the A loop of these peptides are essential for erb B receptor binding. Since the EGF-CFC peptides lack the A loop, this suggests that these proteins probably do not bind directly to any of the known erb B-related tyrosine kinase receptors. Molecular modeling of this modified EGF-like motif in the EGF-CFC proteins has

<table>
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<th>Table 2</th>
<th>Physiochemical properties of EGF-CFC proteins.</th>
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<tr>
<td></td>
<td>Cripto (CR)</td>
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<tr>
<td>Amino acids</td>
<td>188</td>
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<td>$M_r$</td>
<td>21 169</td>
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<td>pl</td>
<td>8.5</td>
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<tr>
<td>DGL</td>
<td>169–171</td>
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<tr>
<td>Glycosylation</td>
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<tr>
<td>N-(Asn)</td>
<td>79–82</td>
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<tr>
<td>NRTCS</td>
<td>S$^{90}$, S$^{91}$</td>
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<tr>
<td>O-(T/S)</td>
<td>S$^{169}$</td>
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<tr>
<td>Phosphorylation</td>
<td></td>
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<tr>
<td>PKC</td>
<td>64–66</td>
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<tr>
<td>169–171</td>
<td>66–68</td>
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<tr>
<td>118–120</td>
<td>76–78</td>
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<tr>
<td>143–145</td>
<td>103–105</td>
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<tr>
<td>PKA</td>
<td>126–129</td>
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<tr>
<td>169</td>
<td>16–19</td>
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<tr>
<td>CK2</td>
<td>34–37</td>
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<tr>
<td>Myristylation</td>
<td>26–31</td>
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<tr>
<td>92–97</td>
<td>120–125</td>
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<tr>
<td>136–141</td>
<td>128–133</td>
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<tr>
<td>179–184</td>
<td>137–142</td>
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</table>

Amino acids are represented by letters.
shown a closely packed three-disulfide stacked arrangement with a disulfide β-cross motif for the first and second disulfides (Lohmeyer et al. 1997). The EGF-motif contains two β-hairpins of six residues in the NH2- and COOH-terminus respectively. In general, the EGF-like motif in the EGF-CFC proteins exhibits less homology to the EGFR ligands and appears to be somewhat more related to structure to the EGF motif in the HRGs that bind to erb B-3 and/or erb B-4.

EGF-CFC proteins range from 171 to 202 amino acids and have a slightly basic pl (Table 2). The unmodified core proteins within this family range from 18 to 21 kDa. Native mouse and human cripto proteins are 24, 28 and 36 kDa in size but additional proteins ranging from 14 to 60 kDa have also been identified by electrophoretic mobility (Brandt et al. 1994, Kenney et al. 1996, Seno et al. 1998, Niemeyer et al. 1998, 1999, Minchiotti et al. 2000). The cryptic protein exists as 20, 21.8 and 23 kDa isoforms while 18 and 20 kDa forms of the oep protein have been found (Shen et al. 1997, Zhang et al. 1998, Gritsman et al. 1999). The variation in size of these proteins as calculated on the basis of electrophoretic mobility may be due to the removal of the hydrophobic signal peptide and to additional posttranslational modifications of the core protein. In this respect, with the exception of oep all the other members of the EGF-CFC family are glycoproteins that contain a single N-glycosylation site and potential O-glycosylation sites. Human cripto-1 was initially expressed in CHO cells as the full-length protein (comprising residues 1–188 of the cDNA sequence (Fig. 1) (Brandt et al. 1994)). Treatment of CR-1–expressing CHO cells with tunicamycin or treatment of cell lysates or conditioned medium with N-glycanase resulted in a shift in the size of the 28 kDa recombinant CR-1 protein to a protein of approximately 21 kDa, the expected size of the core protein (Brandt et al. 1994). A similar effect of N-glycanase was observed with the soluble Cr-1 protein (Minchiotti et al. 2000). Although CR-1 was initially expressed in CHO cells as the full-length protein (comprising residues 1–188 of the cDNA sequence) (Fig. 1), we found that the majority of this protein was retained in the cell as an insoluble form and was subsequently difficult to purify. Studies on the zebrafish CR-1 ortholog, one-eyed pinhead (oep) indicated that the COOH-terminal region may contain a putative GPI-anchorage site (Zhang et al. 1998, Gritsman et al. 1999) and removal of the COOH-terminal stretch generated a soluble form of oep (Gritsman et al. 1999). Consequently, we transiently expressed in CHO cells a COOH-terminally truncated form of CR-1 (CR-1C) (Fig. 3) comprising residues 1–169 of the protein sequence. The human CR-1ΔC protein was efficiently secreted into the supernatant and purified from the conditioned media by chromatography on SP Sepharose, Superose 12 gel-filtration and heparin-Sepharose. Subsequently, the recent characterization of mouse Cr-1 confirmed the presence of a GPI-modification within the COOH-terminal region that is involved in anchoring Cr-1 to the cell membrane (Minchiotti et al. 2000), and that removing this COOH-terminal stretch of residues generates Cr-1 forms that are soluble. On SDS-PAGE under reducing conditions, the human CR-1AC protein migrated as two bands with molecular masses of ~28 kDa. NH2-terminal sequencing revealed that CR-1AC starts with Leu-31, consistent with predictions for processing of the signal peptide using the SIGNALP program (SWISS PROT DATA BASE). Biochemical characterization by peptide mapping, mass spectrometry and glycosidase treatment identified Asn-79 as being an N-linked glycosylation site with >90% occupancy, and Ser-40 and Ser-161 as being O-linked glycosylation sites with 80% and 40% occupancy respectively. In addition, we found that Thr-88 is modified with a single O-linked fucose (Foley S, Williams K & Sanicola M, unpublished data). The O-linked fucose modification is rare and has been shown to occur exclusively within EGF-like modules (Harris & Spellman 1993), and a consensus site has been identified (Harris et al. 1993) as CXXGGS/TC, where the site is located between the second and third conserved cysteines of the EGF module. This consensus site is present in all EGF-CFC proteins, but not in the soluble EGFrerb B-ligands such as EGF, HRG-α or TGFβ that bind to members of the erb B-related type I growth factor receptor family (Fig. 4). There is growing evidence that the presence of fucose groups within functionally important EGF modules is unique to these domains and is a biologically significant feature. EGF-like modules are usually found on secreted proteins involved in blood coagulation or clot dissolution such as blood coagulation factor VII (Kao et al. 1999) and urokinase plasminogen activator (uPA) (Rabbani et al. 1992). O-Fucosylation of uPA within its EGF-module does not affect the affinity of uPA for binding to its receptor but is critical for signaling (Rabbani et al. 1992). Recently, this O-linked fucosylation was shown to be present on the EGF-modules of mammalian Notch 1, and this protein was identified as the first membrane-associated protein with an O-linked fucose (Moloney et al. 2000). Indeed, in one case of the human disease CADASIL, a mutation in Notch 3 disrupts the O-linked fucosylation site, which may influence receptor-ligand interactions (Joutel et al. 1996). Although EGF-CFC family members and the EGF ligands both belong to the EGF superfamily, the presence of a consensus fucosylation site within all EGF-CFC family members and the identification of a fucose modification on CR-1 suggests that the EGF domain in CR-1 more closely resembles the EGF module subfamily and is a biologically important modification that is functionally distinct from the EGF-ligands that bind to the type 1 growth factor receptors. Assessment of the predicted protein products using the PROSITE data base of protein motifs suggests that all of the EGF-CFC proteins also contain several potential
**Figure 2** Ribbon schematic of the predicted and known NMR solution structures of human CR-1 (CRIPTO) and human EGF. Cysteine intramolecular disulfide bridges (yellow) contain three loops: A, red; B, pink; and C, green.

**Figure 3** Sequence of recombinant human CR-1 expressed in CHO cells. Human CR-1 (comprising residues 1–169) was expressed in CHO cells. Cysteines are shown in bold. The fucosylation consensus site is underlined. Domains are color coded to sequences.
myristylation sites which may contribute to their membrane localization (Brandt et al. 1994, Minchiotti et al. 2000). Native or recombinant mouse Cr-1 is a membrane-bound protein anchored by a GPI-linkage to the lipid bilayer (Minchiotti et al. 2000). CR-1, Cr-1, cryptic and oep but not FRL-1, also contain potential GPI sites for cleavage and attachment that are present in the COOH-terminus. In this regard, COOH-truncation by mutagenesis of Cr-1 by 16 amino acids or of oep by 15 amino acids results in proteins that are secreted and, as shown by oep, still retain their biological activity (Zhang et al. 1998, Gritsman et al. 1999, Minchiotti et al. 2000). In addition, Cr-1 protein can also be released by treatment of cells with phosphatidylinositol-specific phospholipase C (PI-PLC) suggesting that the EGF-CFC proteins may function both in a cell-autonomous fashion as GPI-linked, juxtacrine factors and, under certain conditions in which PI-PLC might be activated as soluble, autocrine or paracrine factors in a non-autonomous manner (Gritsman et al. 1999, Minchiotti et al. 2000). Finally, all of the EGF-CFC proteins have the potential to be modified at specific serine and threonine residues by phosphorylation through the action of either protein kinase (PK) C, PKA or casein kinase-2 (CK2).

**EGF-CFC genes in embryonic development**

Vertebrate embryonic development is specified after fertilization by both maternal and zygotic genes. This results in an orchestrated set of morphogenetic movements and differentiation to generate specific sets of cell lineages (Beddington & Robertson 1999, Fraser & Harland 2000). Inductive interactions between cells of different lineages are equally important for the generation of tissues and are mediated by soluble or cell-associated growth factors and morphogens and intracellular (e.g. sprouty) or extracellular antagonists (Table 3) (Kimelman et al. 1992, Beddington & Smith 1993, Beddington & Robertson 1999, Vogel & Gerster 1999, Fraser & Harland 2000, Thissie et al. 2000). Members of the TGFβ (nodal, activin and bone morphogenic proteins (BMPs)), Wnt, Hedgehog (Sonic Hedgehog (Shh)) and FGF (fgf-4 and fgf-8) families are important in controlling positional information, differentiation, patterning and cell movements during early embryonic development in vertebrates (Kimelman et al. 1992, Beddington & Smith 1993, Cornell & Kimelman 1994, Ungar et al. 1995, Furthauer et al. 1997, Isaacs 1997, McGrew et al. 1997, Rossant et al. 1997, Burdsal et al. 1998, Dale & Jones 1999, Kofron et al. 1999, McDowell & Gurdon 1999, Rossant et al. 1997, Burdsal et al. 1998, Dale & Jones 1999, Kofron et al. 1999, McDowell & Gurdon 1999, Vogel & Gerster 1999, Wicking et al. 1999, Agius et al. 2000, Christian 2000, Gritsman et al. 2000, Nishita et al. 2000, Schier & Shen 2000, Thissie et al. 2000). Antagonistic binding proteins such as follistatin, noggin, chordin, antivin and cerberus for specific members within the TGFβ family and antagonists for the Wnt family such as cerberus, frizzled-related protein (FRP) and dickkopf-1 (dkk-1) are also expressed during early embryogenesis (Piccolo et al. 1996, 1999, Moon et al. 1997, Capdevila & Belmonte 1999, Meno et al. 1999, Smith 1999, Thissie & Thissie 1999, Belo et al. 2000, Hashimoto et al. 2000). These antagonists are essential in the generation of morphogenetic gradients which are instrumental in controlling different spatial and temporal aspects of various developmental processes. Finally, transcription factors such as tcf-4, Smad-2 and T-box genes (e.g. Brachyury and Eomesodermin) are either part of a regulatory pathway engaged by these growth factors/morphogens (e.g. tcf-4/wnt-1–β-catenin signaling pathway; Smad-2/activin and nodal signaling pathway) or their expression is regulated by these factors (e.g. Brachyury/ activin and fgf-8) (Smith et al. 1991, Tadano et al. 1993, Yasuo & Satoh 1993, Herrmann & Kispert 1994, Schulte-Merker et al. 1994, Haegel et al. 1995, Huber et al.
Table 3 Factors regulating early vertebrate gastrulation and left-right asymmetry.

<table>
<thead>
<tr>
<th>Growth factors and morphogens</th>
<th>Function</th>
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<tbody>
<tr>
<td>Activins (A and B)</td>
<td>Dorsalization of mesoderm, L/R symmetry</td>
</tr>
<tr>
<td>BMP-4</td>
<td>Ventralization of mesoderm, L/R symmetry</td>
</tr>
<tr>
<td>Nodal (Squint and Cyclops)</td>
<td>Anterior/posterior patterning and axis positioning</td>
</tr>
<tr>
<td>Fgf-3</td>
<td>Ventralization of mesoderm, EMT and mesoderm</td>
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<tr>
<td>Fgf-4</td>
<td>Migration, L/R symmetry</td>
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<tr>
<td>Fgf-8</td>
<td></td>
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<tr>
<td>EGF–CFC peptides</td>
<td>Required for nodal signaling, mesoderm migration</td>
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<tr>
<td>wnt-1</td>
<td>Cooperate with activins for dorsalization of mesoderm and with FGF for ventralization of mesoderm</td>
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<td>wnt-3</td>
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<td>wnt-8</td>
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<tr>
<td>Sonic Hedgehog (Shh)</td>
<td>Induces wnts and nodal, L/R symmetry</td>
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<tr>
<td>Co-stimulators</td>
<td>TGFβ and wnts</td>
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<td>HSPGs (Syndecans)</td>
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<td>Antagonists</td>
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<td>BMP and activin</td>
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<td>Dorsalization of mesoderm</td>
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<tr>
<td>Lef/tcf-4</td>
<td>Induces nodal, c-myc, cyclin-D1</td>
</tr>
<tr>
<td>Smad-2/Smad-4</td>
<td>Mesoderm formation, L/R symmetry</td>
</tr>
<tr>
<td>T-box genes (Brachyury, Eomesodermin and Tbx6)</td>
<td>Posterior mesoderm formation and migration, induces activin and cadherins</td>
</tr>
<tr>
<td>Cubitus interruptus (Ci)</td>
<td>Shh signalling</td>
</tr>
</tbody>
</table>

Gastrulation and germ layer formation

Gastrulation establishes the body plan of the embryo through the formation of the ectoderm, mesoderm and endoderm germ layers (Beddington & Robertson 1999, Camus & Tam 1999, Kodjabachian et al. 1999, Massague & Chen 2000). Spatial positioning of the germ layers results in the establishment of polarity along the anterior/posterior (A/P) axis of the embryo. After the A/P axis and the dorsal/ventral (D/V) axis are established, the left/right (L/R) axis is formed and this eventually leads to the asymmetric development of different tissues and organ systems. These complex events are regulated by Spemann’s organizer (e.g. node/mouse) which temporally expresses different morphogens that can initiate regional differentiation, cell movements and axial positioning (Camus & Tam 1999, Kodjabachian et al. 1999). The first inductive event is the formation of the mesoderm from adjacent ectoderm along the primitive streak. As posterior ectodermal epiblast cells converge towards the primitive streak, they ingress and migrate between the ectoderm and endoderm. Presumptive mesodermal cells migrate through and away from the primitive streak and undergo an epithelial to mesenchymal transition (EMT) (Hay 1995, Viebahn 1995, Camus & Tam 1999). During EMT, cell-cell contacts are disrupted with a loss of expression of the adherens junction protein, E-cadherin (Hay 1995, Guarino et al. 1999). Concomitantly, cytokeratin expression is reduced and the expression of intermediate filament proteins...
proteins such as vimentin is up-regulated (Thiery & Chopin 1999). These events are quite analogous to events which occur during the invasive stages of tumor cell metastasis where tumor epithelial cells become motile and assume more mesenchymal characteristics (Guarino et al. 1999, Thiery & Chopin 1999).


In zebrafish, two mouse nodal-related genes squnt (sqt) and cyclops (cyc) are essential for mesoderm and endoderm formation and for A/P axis development (Feldman et al. 1998, Rebagliati et al. 1998, Sampath et al. 1998, Gritsman et al. 2000, Schier & Shen 2000). Expression of zygotic genes such as nodal/sqt and cyc and fgf-8 are, in turn, regulated by a maternally-derived T-box gene (e.g. VegT) (Kofron et al. 1999, Agius et al. 2000, Schier & Shen 2000). Similar to sqt and cyc, zebrafish oep is also necessary for initiating mesoderm, endoderm and A/P axis formation (Schier et al. 1997, Zhang et al. 1998, Gritsman et al. 1999, 2000, Schier & Shen 2000). In this context, oep is an essential component for sqt and cyc signaling and functions in a cell-autonomous role as a possible co-receptor or competence factor for nodal-related proteins but not for activin (Gritsman et al. 1999, Schier & Shen 2000). Nodal-related proteins and oep subsequently depend upon and/or signal through the downstream serine/threonine kinase activin type IIB and type IB receptors that engage intracellular Smad-2 and Smad-4 phosphorylation and transcriptional activation (Peyrieras et al. 1998, Schier & Shen 2000). Antivin, a lefty-related factor, and cerberus are competitive antagonists of activin and nodal for binding to the type IIB activin receptor in this pathway (Thirse et al. 2000). Mutations in oep result in cyclopia, absence of head and trunk mesoderm, loss of prechordal plate and ventral neuroectoderm, impairment of gastrulation movements and loss of A/P axis patterning and positioning (Zhang et al. 1998, Gritsman et al. 1999). Rescue of the oep mutant phenotype can be achieved by expression of full-length or secreted COOH-terminal truncated oep protein suggesting that oep can function under certain conditions as a paracrine effector. Ectopic expression of Xenopus FRL-1 or mouse Cr-1 or overexpression of activin or activation of downstream components in an activin-like signaling pathway such as the activin type IB receptor or Smad-2 can also rescue the mutation (Gritsman et al. 1999). However, rescue of the mutant phenotype cannot be achieved with BMP-2 or -4, activated ras, mitogen-activated protein kinase (MAPK) or with different members in the wnt/β-catenin signaling pathway (Gritsman et al. 1999, Schier & Shen 2000).

In Xenopus, there is some indication that FRL-1 function during development may depend upon a functional FGFR signaling pathway since this gene was initially identified using a functional screen in yeast to detect novel ligands of the FGFR-1 (Kinoshita et al. 1995). In Xenopus oocytes and in yeast (Saccharomyces cerevisiae), simultaneous ectopic overexpression of FRL-1 and the Xenopus FGFR-1 kinase leads to enhanced Ca\(^{2+}\) influx and to an increase in the indirect transphosphorylation of tyrosine on and transactivation of the FGFR-1. However, no direct binding of FRL-1 to the Xenopus FGFR-1 could be detected. In isolated animal caps from Xenopus embryos, FRL-1 similar to FGF-2, activin, Xenopus-nodal (Xnr) or the Xwnt-8 induces the formation of mesoderm and the expression of mesoderm-related genes. In the case of FRL-1, this occurs in a paracrine, non-cell-autonomous manner through an FGFR-1-mediated pathway. Overexpression of FRL-1 in Xenopus embryos leads to the preferential development of posterior mesoderm-related structures at the expense of anterior head structures. FRL-1 expression in Xenopus is detected during gastrulation and during the early stages of neurulation.

During early mouse embryogenesis, Cr-1 expression can first be detected by RT-PCR in the inner cell mass (ICM) of the blastocyst at day 4 of development, which seems logical
since CR-1 and Cr-1 were first identified in undifferentiated human NTERA2/D1 and mouse F9 embryonal carcinoma cells respectively (Ciccodicola et al. 1989, Dono et al. 1993, Johnson et al. 1994, Ding et al. 1998, Xu et al. 1999). Expression of both genes is lost in NTERA2/D1 and F9 cells following retinoic acid-induced differentiation (Ciccodicola et al. 1989, Dono et al. 1993, Minchiotti et al. 2000). This expression pattern is clearly distinct from that of cryptic which is only found in differentiated mesoderm cells following retinoic acid treatment and not in embryonal carcinoma or ICM stem cells (Shen et al. 1997). Cr-1 mRNA expression in the embryo is found in the embryonic ectoderm following implantation of the blastocyst. Expression increases on day 6.5 of gestation which is the point where gastrulation begins with the formation of the primitive streak (Dono et al. 1993, Johnson et al. 1994, Ding et al. 1998, Xu et al. 1999). Expression then decreases dramatically by day 7. By in situ hybridization, expression of Cr-1 mRNA in the midgestation mouse embryo is temporally and spatially restricted to the epiblast cells of the primitive streak, to the developing mesoderm during gastrulation and in later fetal stages to the myocardium of the truncus arteriosus of the developing heart. In the developing heart, Cr-1 mRNA can be detected in the epimyocardium but not in the endocardium. With the exception of the developing heart, little if any expression of Cr-1 mRNA can be detected in the remainder of the embryo after day 8. Expression of Cr-1 mRNA in adult tissues is generally several-fold less than in undifferentiated F-9 mouse embryonal carcinoma cells (Dono et al. 1993, Minchiotti et al. 2000). Cr-1 protein expression in the developing embryo was also assessed by immunocytochemistry using a rabbit antibody generated against the full-length mouse Cr-1 protein (Minchiotti et al. 2000). Cr-1 mRNA and immunoreactive protein were found to co-localize in the developing mesoderm of the 6.5 day embryo while at day 8.5 Cr-1 protein expression was detected in the myocardium of the developing heart (Minchiotti et al. 2000).

The expression pattern for cryptic is distinct from the expression pattern for Cr-1 since it is not expressed in adult mouse tissues and has a partially overlapping yet distinct expression pattern to Cr-1 in the midgestation mouse embryo (Shen et al. 1997, Yan et al. 1999). Cryptic expression can first be detected in the axial mesoderm and then becomes more restricted to the anterior end of the primitive streak and the head process. Expression is absent in the prechordal plate but is present in the lateral plate mesoderm, node and floor plate of the neural tube. After day 8.5, little expression of cryptic mRNA can be detected in the embryo.

**Left-right axis formation and cardiac development**


Mouse Cr-1 may also perform an essential role during earlier stages of cardiac lineage specification and differentiation. Homologous recombination that leads to homozygous knockout of the Cr-1 gene (Cr-1<sup>−/−</sup>) in pluripotent embryonic stem cells (ES cells) impairs their ability spontaneously to differentiate in vitro into cardiomyocytes without affecting the ability of ES cells to differentiate into other cell types (Xu et al. 1998).

Interestingly, a similar embryonically lethal phenotype due to myocardial and endocardial defects is found in day 10 embryos from erb B-4, erb B-2 and NRG1 knockout mice (Klapper et al. 2000). This may have functional significance since the human CR-1 protein can indirectly increase the tyrosine transphosphorylation of erb B-4 and may depend upon erb B-4 for signaling in certain cells via heterodimerization with or indirect cross-talk to the CR-1 receptor (Bianco et al. 1999). Germline knockout of the mouse Cr-1 gene is embryonically lethal as embryos die in utero between 8.5 and 9.5 days of development due to their inability to gastrulate and form appropriate germ layers as a consequence of the complete absence of the primitive streak (Ding et al. 1998, Xu et al. 1999). Specifically, disruption of Cr-1 in Cr-1<sup>−/−</sup> embryos results in the formation of embryos...
that possess a head without a trunk demonstrating that there is a severe deficiency in embryonic mesoderm and endoderm without a loss of anterior neuroectoderm formation. Therefore, Cr-1 signaling is necessary for cell movements that correctly orient the embryo along the A/P axis and for cell migration of the presumptive developing mesoderm and for reorientation of the endoderm (Ding et al. 1998, Xu et al. 1999, Schier & Shen 2000). Chimeric embryos that were generated between wild-type and Cr-1−/− ES cells were normal indicating that Cr-1 produced by the wild-type host cells could rescue the mutant phenotype in a paracrine manner (Xu et al. 1998). Fibroblasts that were derived from Cr-1−/− embryos were found in vitro to exhibit a ∼50% reduction in growth rate as compared with embryonic fibroblasts from wild-type embryos. In addition, Cr-1−/− fibroblasts exhibited negligible migration in a wound healing assay and were impaired in their ability to migrate over or chemotactically towards either fibronectin or type 1 collagen as compared with embryonic fibroblasts from wild-type embryos (Xu et al. 1999).

**Biological effects of cripto in differentiated mammalian cells**

**In vitro effects of transduced Cr-1 or recombinant CR-1 in mammary epithelial cells**

In rat CREF embryo fibroblasts or rat FRTL-5 thyroid epithelial cells that have been transformed by either an activated c-Ha-ras or c-Ki-ras proto-oncogene respectively, expression of rat Cr-1 is substantially up-regulated (Su et al. 1993, Mincione et al. 1998). Reversion of the transformed phenotype of several of the Ha-ras-transformed CREF clones by overexpression of the Krev-1 ras suppressor gene results in a loss of expression of rat Cr-1, suggesting that Cr-1 might function as a proximal effector of p21ras transformation in this system and might contribute to cellular transformation. In fact, the first indication that cripto had any biological activity in a mammalian cell was demonstrated by the ability of a transduced human CR-1 or mouse Cr-1 cDNA to transform mouse NIH/3T3 fibroblasts, mouse NOG-8 and mouse CID-9 mammary epithelial cells when the genes were overexpressed in these cells in vitro (Ciardiello et al. 1991a, Niemeyer et al. 1998). Cripto-transduced mammary epithelial cells were able to grow in soft agar. In monolayer culture, the cripto-transduced cells exhibited an enhanced proliferation rate, clonogenic potential and a loss of contact growth inhibition. However, NOG-8 or CID-9 transformants were unable to form tumors in nude mice, suggesting that additional genetic alterations were necessary to complete the tumorigenic phenotype in vitro (Ciardiello et al. 1991a, Niemeyer et al. 1998). Reciprocally, knockout of the Cr-1 gene in CID-9 cells reduced growth and increased apoptosis in these cells in vitro and in vivo. Overexpression of the mouse Cr-1 protein in primary FVB/N or C57Bl/6 mouse mammary epithelial cells using a recombinant amphotropic retroviral expression vector containing the Cr-1 cDNA facilitated lateral ductal branching and clonal expansion of mammary ductal hyperplasias after these retrovirally transduced cells were reintroduced into the cleared mammary fat pad of syngeneic, ovariectomized virgin mice (Kenney et al. 1997a). These ductal hyperplasias could be serially transplanted in the mammary fat pad, demonstrating that they represent an immortalized population of mammary epithelial stem cells. However, frank mammary carcinomas have not been observed in these hyperplastic outgrowths even after several in vivo transplantations.

When inserted into the fourth inguinal mammary gland of ovariectomized virgin mice, slow-release Elvax pellets containing the p47 EGF-like motif of the CR-1 protein produce a 20-fold increase in DNA synthesis in the adjacent ductal epithelial cells. The same increase is seen in terminal end buds (TEBs) that immediately surround these pellets relative to the labeling index and branching in epithelial cells that surround Elvax pellets containing only bovine serum albumin (Kenney et al. 1997a). In addition, a threefold increase in secondary and tertiary lateral ductal branching in areas surrounding these CR-1 pellets occurs in nearly 70% of the mammary outgrowths. A comparable in vitro-related effect has been observed with recombinant CR-1 or with cells transduced with a Cr-1 cDNA expression vector in mouse NMuMG, TAC-2 and EpH4 mammary epithelial cells that are grown in type 1 collagen gels (C Wechselberger, A D Ebert, C Bianco, B Wallace-Jones, N Khan, Y Sun & D S Salomon, unpublished data). Despite having a growth-promoting effect when overexpressed, Cr-1 also reduced contact inhibition in monolayer culture. In addition, induction of anchorage-independent growth was achieved in the Cr-1-transfected EpH4 cell line. More importantly, all the transfected, Cr-1 overexpressing cell lines exhibited an increase in migratory potential as assessed in various assays. The cells showed increased scattering on matrigel and enhanced chemotaxis in Boyden chamber and migration in wound healing assays. Recombinant CR-1 is also able to induce the scattering of NOG-8 mouse mammary epithelial cells grown at low densities as colonies on plastic. This effect is probably related to an increase in cell motility that is induced by this peptide either through chemokinesis or through chemotaxis. When cultured in a three-dimensional type-1 collagen gel matrix, the Cr-1–transfected cells formed branching tubules.

The ability of Cr-1 to stimulate both scattering and branching morphogenesis in collagen gels is reminiscent of the effects induced by hepatocyte growth factor (HGF) or TGFβ1 in mammary epithelial cells (Santos & Nigam 1993, Montesano et al. 1997). The scattering effect is preceded by a change in morphology of these epithelial cells to a more fibroblastic-like phenotype. A similar effect on motility and
EMT can be produced in these cells by HGF but not by HRGβ1, which is only mitogenic. EMT is often characterized by a decrease in cell-cell adhesion which coincides with enhanced migration. In this respect it was reported that tyrosine phosphorylation of β-catenin can inhibit its association with E-cadherin and facilitate cell scattering and migration (Shibamoto et al. 1994). Cr-1 overexpressing cell lines exhibit an enhanced basal level of tyrosine phosphorylation of β-catenin, resulting in a decreased association of β-catenin to E-cadherin. This effect could only be observed in the presence of the tyrosine phosphatase inhibitor, sodium orthovanadate, which indicates a very stringent regulation of this process in mammary epithelial cells.

HC-11 mouse mammary epithelial cells express the milk protein β-casein at confluency after exposure to the lactogenic hormone mixture of dexamethasone, insulin and prolactin (DIP) (Ball et al. 1988, Hynes et al. 1990). Similar to other EGF-related peptides that bind to the EGF receptor (EGFR) or peptides that can bind to either c-erb B-3 or c-erb B-4, CR-1 can function as a competence factor and can prime HC-11 cells that have been pretreated during logarithmic growth to respond optimally to subsequent treatment with DIP for the induction of expression of β-casein (De Santis et al. 1997). In contrast, the continuous presence of EGF, TGFα, or HRGβ1 can actually inhibit DIP-mediated transcription of the β-casein gene presumably due to the inactivation of the transcription factor Stat-5, which is activated by prolactin receptor engagement (Hynes et al. 1990, Taverna et al. 1991, Yang et al. 1994, Marte et al. 1995). Similar to EGF, the simultaneous presence of CR-1 with DIP was found to effectively block DIP induction of β-casein protein expression in a dose-dependent fashion (De Santis et al. 1997). In addition, HC-11 cells which are infected with a recombinant amphotropic retroviral expression vector containing the mouse Cr-1 cDNA and which continuously overexpress the Cr-1 protein also exhibit low levels of β-casein protein expression following DIP treatment. The inhibitory effect of CR-1 is not unique to HC-11 cells since a similar inhibitory effect on β-casein expression in the presence of DIP was also observed in CID-9 cells that are overexpressing the Cr-1 gene (Niemeyer et al. 1998).

To ascertain if CR-1 could alter the expression of additional milk proteins in response to lactogenic hormones, mammary explant cultures from midpregnant mice were maintained in serum-free medium with various peptides in the absence or presence of DIP (De Santis et al. 1997). There was little β-casein expression in primary explants prior to culture or in explant cultures maintained in the absence or presence of different EGF-related peptides. However, inclusion of DIP alone into the medium was able fully to induce β-casein expression. Concurrent treatment with EGF, TGFα, HB-EGF, AR, BTC or CR-1 was able significantly to impair to different degrees DIP-induced expression of β-casein in primary mouse mammary explant cultures. Expression of whey acidic protein (WAP) in these primary mammary epithelial explant cultures was also induced by DIP, and this response to DIP is also significantly attenuated to different degrees by these various EGF-related peptides including CR-1. In contrast to growth factors that activate the EGFR or the CR-1 receptor, HRGβ1 stimulates the expression of β-casein and WAP in these DIP-treated midgestation mammary explant cultures. α-Lactalbumin expression following DIP treatment was generally less sensitive to the modulatory effects of these peptides. The differential regulatory effects of these structurally similar peptides may be related to the normal temporal sequence of lactogenic hormone-induced activation of early (β-casein) and late (WAP and α-lactalbumin) milk protein genes that occurs during pregnancy in mammary alveolar epithelial cells and/or to the combination or sequence of activation of different members of the erb B type 1 receptor tyrosine kinase family expressed in these cells.

The inhibitory effect of exogenous CR-1 on β-casein and WAP expression may be biologically significant since endogenous Cr-1 expression is up-regulated in alveolar cells during pregnancy and lactation and therefore may function as a negative modulator to initiate a reduction in milk protein expression as the mammary gland enters involution when the secretory epithelial cells begin to undergo apoptosis (Kenney et al. 1995, Herrington et al. 1997). This may be the case since we have found recently that CR-1 can induce apoptosis in several nontransformed mouse mammary epithelial cell lines. Apoptosis could be observed after 2 days exposure of confluent HC-11 cells to CR-1 when cells were deprived of the survival factors, EGF and insulin (De Santis et al. 2000). Apoptosis was mediated through the induction of a caspase-3-like protease and down-regulation in the expression of the anti-apoptotic protein Bcl-xL. The induction of apoptosis under reduced serum by CR-1 was preceded by an inhibition in cell growth and a reduction in poly(ADP-ribose) polymerase (PARP) protein and an increase in β-catenin cleavage. A reduction in β-catenin levels due to caspase-3-mediated degradation may result in a loss of cell-cell and cell-matrix interactions with an increase in anoikis (De Santis et al. 2000). Cr-1 has also been demonstrated to act as a survival factor in CID-9 cells transfected with a Cr-1 expression vector when cells were propagated under sparse, subconfluent conditions in medium containing low serum and insulin (Niemeyer et al. 1998).

**Expression of mouse Cr-1 in the mammary gland and in mammary tumors**

Expression of Cr-1 in the developing mouse mammary gland has been examined by RT-PCR, in situ hybridization, immunocytochemistry and Western blot analysis (Kenney et
Different levels of Cr-1 expression are found in the virgin, pregnant, lactating, involving and aging mammary gland. In the virgin prepubescent mammary gland, a 26 kDa immunoreactive Cr-1 protein and Cr-1 mRNA are observed primarily but not exclusively in the cap stem cells of the growing terminal end buds (Kenney et al. 1995). As assessed by Western blot analysis, immunocytochemistry and in situ hybridization, expression of Cr-1 in both ductal and lobuloalveolar cells is enhanced by approximately three- to fivefold during pregnancy and lactation and is totally lost during involution (Kenney et al. 1995, Herrington et al. 1997, Niemeyer et al. 1998). Enhanced expression of Cr-1 may be driven by lactational hormones. In this respect CID-9 cells, which like HC-11 cells, were established from Comma-1D cells that were isolated from a mid-pregnant Balb/c mouse express Cr-1 (Niemeyer et al. 1998). Expression of Cr-1 in these cells can be enhanced when the cells are stimulated to grow and differentiate in response to DIP on a reconstituted basement membrane (Niemeyer et al. 1998). This may be of physiological significance since immunoreactive and bioactive CR-1 has recently been detected in the milk from several different human donors (Bianco et al. 2000). Interestingly, Cr-1 expression is elevated to an even greater extent in ductal epithelial cells in the aging (2–year-old) mammary gland of nulliparous or multiparous Balb/c mice which exhibit spontaneous mammary tumor development (Herrington et al. 1997, Kenney et al. 1997b).

Cr-1 mRNA and protein are generally overexpressed in a majority of mouse mammary carcinoma cells relative to noninvolved adjacent mammary epithelium in several different spontaneous and carcinogen-induced mouse mammary tumors (Herrington et al. 1997). In addition, Cr-1 overexpression has also been observed in mammary tumors and in their lung metastases arising in transgenic mice that overexpress different transforming oncogenes such as TGF-erb B-2, erb B-2, int-3, polyoma middle T antigen or SV40 large T antigen (Kenney et al. 1996, Niemeyer et al. 1999). In mouse mammary tumor virus (MMTV)-polyoma middle T transgenic FVB/N mice, Cr-1 protein expression in the mammary gland could first be detected in day 44 virgin mice where levels were seven- to tenfold higher than in the mammary gland from comparably aged control FVB/N virgin female mice (Niemeyer et al. 1999). At 44 days, this expression is detected in ductal hyperplasias. Tumors developing from these hyperplasias showed levels of Cr-1 protein 25–fold higher than in the mammary gland from non-transgenic mice. Enhanced expression of Cr-1 was also detected in hyperplasias from MMTV-erb B-2 and metallothioinein (MT)-TGF-erb B-2 transgenic mice suggesting that expression of Cr-1 is an early marker for premalignancy in the mammary gland of these animals (Niemeyer et al. 1999). Compared with the lactating mammary gland that expresses only 18 and 26 kDa isoforms of Cr-1, mammary tumors that arise in different transgenic animals overexpress several distinct and specific immunoreactive isoforms of the Cr-1 protein ranging from 14 to 60 kDa (Kenney et al. 1996). These proteins may arise by alternative mRNA splicing, posttranslational modifications (e.g. glycosylation or myristylation) or proteolytic processing.

Effect of CR-1 on human cervical carcinoma cells in vitro

Alterations in the behavior of tumor cells such as cell migration and invasion correlate with changes in the composition or structure of the intermediate filament network (Goldman et al. 1996, Hendrix et al. 1996). The intermediate type-III filament protein, vimentin, is generally expressed in mesenchymal cells and is overexpressed in several different human carcinomas (Raymond & Leong 1989, Fuchs & Weber 1994). Vimentin expression is associated with increased invasiveness and motility of primary human cervical cancer cells in vitro (Gilles et al. 1994, 1996, 1999). Caski cervical carcinoma cells which normally express only low levels of vimentin were stably transfected with an expression vector containing the human CR-1 cDNA. Changes in the expression pattern of several genes were analyzed with the cDNA expression array technique (Ebert et al. 2000). Genes found to be either up- or down-regulated in the CR-1 overexpressing Caski cells relative to Caski cells transfected with the vector alone are summarized in Table 4. Gene expression changes of more than threefold are indicated in bold letters. As compared with vector-transfected Caski cells, a threefold increase in vimentin gene expression was observed in the CR-1-transfected Caski cells as verified by Northern blot analysis. In cell lysates of CR-1-transfected Caski cells, a sixfold increase in vimentin protein expression was detected by Western blot analysis as compared with vector-transfected cells which correlated with the cDNA expression array data. These results were verified by treating wild-type Caski cells with exogenous, recombinant CR-1 for 24 h. At this time, a 3.5-fold upregulation of vimentin protein expression was detected by Western blot analysis. To assess the migratory potential of CR-1-overexpressing and CR-1-treated Caski cells, cell motility was quantified in the Boyden chamber assay. CR-1-transfected Caski cells exhibited a significant increase in their invasive capacity with respect to migration through collagen- or gelatin-coated polycarbonate membranes.

CR-1 may also function as a survival factor in human carcinoma cells. For example, transfection and overexpression of human CR-1 in SiHa and Caski human cervical carcinoma cells can enhance the survival of these cells under serum-restricted culture conditions (Ebert et al. 1999). Apoptosis can be initiated by exposure of these cervical...
Table 4 Genes that are modulated by Cripto-1 in human cervical cancer. Genes that are highlighted in bold represent an increase or decrease in expression by at least threefold as detected by use of a Clontech Human Tumor Atlas Array.

<table>
<thead>
<tr>
<th>Induced</th>
<th>Repressed</th>
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<tbody>
<tr>
<td>p21 CIP/WAF</td>
<td>IGFBP-3</td>
</tr>
<tr>
<td>GADD153</td>
<td>IGFBP-5</td>
</tr>
<tr>
<td>GADD45</td>
<td>p16INK</td>
</tr>
<tr>
<td>Cyclin D2</td>
<td>p14 CDK inhibitor</td>
</tr>
<tr>
<td>GDN</td>
<td>Keratin 2</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Keratin 13</td>
</tr>
<tr>
<td>Integrin α5</td>
<td>Integrin β4</td>
</tr>
<tr>
<td>Integrin α6</td>
<td>γ-Catenin</td>
</tr>
<tr>
<td>Tenascin</td>
<td>FGF-5</td>
</tr>
<tr>
<td>Rho 8</td>
<td>FGF-6</td>
</tr>
<tr>
<td>Laminin β1</td>
<td>MMP-2</td>
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<tr>
<td>uPA</td>
<td></td>
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<tr>
<td>Plasminogen</td>
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<tr>
<td>VEGF</td>
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<tr>
<td>HB-EGF</td>
<td></td>
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<tr>
<td>Fibronectin</td>
<td></td>
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<tr>
<td>PAI-1</td>
<td></td>
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<td>PAI-2</td>
<td></td>
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<tr>
<td>MMP-14</td>
<td></td>
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<tr>
<td>IGFBP, insulin like growth factor binding protein; VEGF, vascular endothelial growth factor; GDN glial cell-derived neurotropicin; PAI, plasminogen activator inhibitor; MMP, metalloprotease protein.</td>
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</table>

carcinoma cells to the phosphatidylinositol 3–kinase (PI-3 kinase) inhibitor, LY294002. This drug-induced apoptosis can be partially rescued by treatment of SiHa and Caski cells with recombinant CR-1 suggesting that the PI-3 kinase/Akt pathway may be responsible for this survival activity. In fact, CR-1 expression and enhanced survival is correlated with the ability of CR-1 to induce the tyrosine phosphorylation of the p85 regulatory subunit of PI-3 kinase in these cells and to the subsequent activation of PKB/Akt as assessed by an increase in the phosphorylation of Akt and as measured by an increase in the phosphorylation of the Akt substrate, glycogen synthase kinase-3β (GSK-3β). Since phosphorylation of GSK-3β inactivates the enzyme, which in the wnt signaling pathway results in a reduction in β-catenin degradation, CR-1 may also contribute to the activation of this pathway. This may be significant since cyclin-D1 and c-myc are two oncogenes that are up-regulated by the wnt-activated β-catenin/Tcf transcription complex (Brabletz et al. 2000).

Mechanism of action of EGF-CFC proteins in mammalian cells

Activation of ras/raf/MAPK and PI-3 kinase/Akt/GSK-3β pathways by cripto

In zebrafish, oep functions in a nodal signaling pathway during embryonic development (Gritsman et al. 1999). Since oep is cell-associated and acts predominantly in a cell-autonomous manner, oep might function as a co-receptor for an activin type IIB receptor complex (Schier et al. 1997, Zhang et al. 1998, Gritsman et al. 1999). Although activin type IIB receptors are expressed in adult mammalian cells, nodal expression has not yet been detected in a fully differentiated mammalian cell. In addition, there is no source for recombinant nodal protein to ascertain its potential interaction with other EGF-CFC proteins in various mammalian cell types. Nevertheless, recent experiments with mouse mammary HC-11 cells have demonstrated that recombinant CR-1 is able to enhance the tyrosine phosphorylation of the SH2–adapter protein Shc leading to the subsequent downstream activation of the ras/raf/MAPK signaling pathway (Kannan et al. 1997). It was shown that exogenous CR-1 protein binds to high affinity, saturable binding site(s) on HC-11 cells as well as on several different human breast cancer cell lines. This interaction was highly specific and could not be blocked by ligands for the various erb B receptors such as EGF, TGFα, AR, BTC or HRGβ1. Exogenous CR-1 induced a rapid increase in the tyrosine phosphorylation of p66, p52, and p46 Shc in various cell lines. Furthermore, CR-1 promoted an increased association of the adapter Grb2–SOS signaling complex with tyrosine phosphorylated Shc in HC-11 cells. This cascade led to an increase of MAPK activity demonstrating that CR-1 can function through a receptor which activates intracellular effectors in the ras/raf/MEK/MAPK pathway. In addition to various isoforms of Shc, CR-1 also induced an increase in the tyrosine phosphorylation of other unidentified proteins at 120 kDa, 80 kDa and 60 kDa.

The inhibitory response of CR-1 on β-casein expression after treatment of HC-11 cells with DIP could be attenuated by B-581 which is a specific peptidomimetic farnesyl transferase inhibitor that blocks p21pro farnesylation and activation, or by the PI-3 kinase inhibitors, wortmannin or LY-294002 but not by PD-98059, a mitogen-activated, erk-activating kinase (MEK) inhibitor that blocks MAPK activation (De Santis et al. 1997). These data suggest that the ability of CR-1 to block lactogenic hormone-induced expression of β-casein at least in HC-11 cells is mediated through a p21pro-dependent, PI-3 kinase-mediated pathway. This is further substantiated by the observation that CR-1 is able transiently to stimulate the tyrosine phosphorylation of the p85 PI-3 kinase regulatory subunit and transiently to increase the activity of PI-3 kinase in HC-11 cells. These data collectively demonstrate that CR-1 can function as a growth factor but more importantly as a differentiation factor in mammary epithelial cells during pregnancy in the regulation of milk protein expression. The elevated expression of Cr-1 during pregnancy and its presence in milk supports a potential in vivo role for this growth factor at this stage in mammary gland development.
Transactivation of erb B-4 and FGFR-1 by CR-1

Since EGF-CFC proteins contain a modified EGF-like motif, it seemed possible that these peptides might bind to one or several of the erb B type 1 tyrosine kinase receptors. However, recombinant CR-1 in radioreceptor competition assays with $^{125}$I-EGF in human mammary MCF-10A cells or A431 epidermoid carcinoma cells failed to demonstrate any binding to the EGFR on these cells (Kannan et al. 1997). Reciprocally, $^{125}$I-CR-1 binding to several different human breast carcinoma cells lines could be blocked by CR-1 but not by EGF, TGFα, BTC, AR or HRGβ1. Finally, recombinant CR-1 using BAF/3 mouse lymphoid cells that were ectopically expressing the human EGFR, erb B-2, erb B-3, or erb B-4 either alone or in various pairwise combinations was unable to activate directly the tyrosine phosphorylation of these tyrosine kinase receptors, demonstrating that CR-1 was not able to bind directly to these type 1 receptor kinases either as homodimers or as heterodimers (Kannan et al. 1997).

Ligand binding to various tyrosine kinase receptors can induce tyrosine transphosphorylation between different receptor subtypes leading to an enormous degree of signal diversification through dimerization both within receptors of the same family and in some cases between receptors of different families (Carraway & Cantley 1994, van Weering & Bos 1998). This has been shown to be a common event within the erb B family of receptor tyrosine kinases. However, this has also been documented to occur in the FGFR and platelet-derived growth factor (PDGF) receptor families (Bellot et al. 1991, Turck & Edenson 1994). erb B-2 undergoes transactivation and an enhanced tyrosine phosphorylation in response to both EGF and neu differentiation factor/HRGs which do not bind directly to this receptor but bind either to the EGFR or to the erb B-3 or erb B-4 receptors respectively (King et al. 1988, Sliwkowski et al. 1994, Beerli et al. 1995). Another example of heterodimerization is the interaction with erb B-3. erb B-3 has an impaired tyrosine kinase activity, but it can undergo transactivation and phosphorylation by other erb B members (Guy et al. 1994, Riese et al. 1995). These cross-receptor interactions within the erb B family raised the possibility that although CR-1 does not bind directly to any of the erb B receptors, it may be able indirectly to transactivate members of the erb B receptor family by heterodimerization and transphosphorylation by either its receptor or indirectly through a soluble, cytoplasmic src-like or JAK-like tyrosine kinase. This possibility was investigated in detail and it was shown that CR-1 was able specifically to transactivate erb B-4 in several mouse and human mammary epithelial cell lines whereas it failed to increase the tyrosine phosphorylation of the other three erb B members (Bianco et al. 1999). Blocking of the erb B-4 receptor with a specific neutralizing antibody or inhibiting expression with an erb B-4 antisense expression vector was able to inhibit MAPK activation in response to treatment with recombinant CR-1 in MDA-MB-453 and T47-D human breast cancer cells. These data suggest that erb B-4 although not a direct receptor for this growth factor is required for the full activation of MAPK in a signal transduction cascade that is engaged by CR-1 in some human breast cancer cells. A similar effect on the enhancement of tyrosine transphosphorylation occurs in response to FRL-1 for the FGFR-1 receptor in Xenopus (Kinoshita et al. 1995). In addition, recombinant CR-1 produces a similar effect on specifically enhancing FGFR-1 but not FGFR-2, FGFR-3 or FGFR-4 tyrosine phosphorylation in trans in human breast cancer MDA-MB-134 and T47-D cells (C Bianco, C Wechselberger & D S Salomon, unpublished data). This stimulatory effect occurs in trans since a specific FGFR-1 tyrosine kinase inhibitor, SU-5460, is able to block FGF-2–induced tyrosine phosphorylation but not CR-1–induced phosphorylation of the FGFR-1.

Expression of cripto in human carcinomas and premalignant lesions

Several studies have demonstrated that growth factors are involved in the pathogenesis of human cancer (Goustin et al. 1986). In fact, tumor cells generally exhibit a reduced requirement for exogenous growth factors when compared with normal cells (Aaronson 1991, Sporn & Roberts 1992). This phenomenon may be due to an increase in the synthesis by transformed cells of various growth factors that can regulate tumor growth, angiogenesis and metastasis. CR-1 is overexpressed in a number of human breast, colon, gastric and pancreatic cell lines, suggesting that CR-1 may function as an autocrine growth factor in these tumor cells (Ciardiello et al. 1991a, Kuniyasu et al. 1991, Friess et al. 1994). To assess the role of CR-1 in human malignancies, different primary human carcinomas, premalignant lesions and adjacent noninvolved tissues have been analyzed for the frequency and relative expression of CR-1 mRNA and/or immunoreactive protein (Table 5).

Expression of CR-1 in gastrointestinal malignancies

Gastric cancer

CR-1 can be detected in normal gastric mucosa and in the mature regenerative epithelium of gastric ulcers where it may be involved in ulcer healing, probably through a paracrine mechanism (Abe et al. 1997). An increase in CR-1 expression has been observed in 53% of premalignant lesions of the stomach, such as intestinal metaplasia of the gastric mucosa, in gastric adenomas, and in 46% of early and
Table 5 Expression of immunoreactive CR-1 in human lesions. Data represent number of positive samples/total number of samples analyzed. Numbers in parentheses represent % positive samples.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Noninvolved epithelium</th>
<th>Premalignant lesion</th>
<th>Carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TA</td>
<td>TVA</td>
<td></td>
</tr>
<tr>
<td>Colon</td>
<td>26/65 (40)</td>
<td>10/13 (77)</td>
<td>122/168 (73)**</td>
</tr>
<tr>
<td>Stomach</td>
<td>1/37 (3)</td>
<td>16/30 (46)**</td>
<td>17/37 (46)**</td>
</tr>
<tr>
<td>Pancreas</td>
<td>10/58 (17)</td>
<td>58/98 (59)**</td>
<td>50/98 (59)**</td>
</tr>
<tr>
<td>Gall bladder</td>
<td>N.D.</td>
<td>6/9 (67)</td>
<td>89/132 (68)**</td>
</tr>
<tr>
<td>Breast</td>
<td>5/33 (15)</td>
<td>26/55 (47)</td>
<td>497/631 (79)**</td>
</tr>
<tr>
<td>Non small cell lung</td>
<td></td>
<td></td>
<td>178/195 (91)**</td>
</tr>
<tr>
<td>Ovary</td>
<td>6/7 (86)</td>
<td>6/14 (43)</td>
<td>4/5 (80)</td>
</tr>
<tr>
<td></td>
<td>0/7</td>
<td>4/10 (40)</td>
<td>23/40 (58)</td>
</tr>
<tr>
<td></td>
<td>3/9 (33)</td>
<td>3/8 (38)</td>
<td>25/48 (52)</td>
</tr>
<tr>
<td></td>
<td>4/8 (50)</td>
<td>Borderline: 10/10</td>
<td>9/10 (90)</td>
</tr>
<tr>
<td>Endometrium</td>
<td>10/28 (36)</td>
<td></td>
<td>53/91 (58)*</td>
</tr>
<tr>
<td>Cervix</td>
<td>4/25 (17)</td>
<td></td>
<td>68/96 (71)*</td>
</tr>
<tr>
<td>Testis</td>
<td>0/3</td>
<td></td>
<td>40/74 (54)*</td>
</tr>
<tr>
<td>Adrenal cortex</td>
<td>1/3 (33)</td>
<td></td>
<td>29/51 (57)**</td>
</tr>
<tr>
<td>Bladder</td>
<td>0/6</td>
<td></td>
<td>23/39 (60)**</td>
</tr>
<tr>
<td>Renal</td>
<td>0/18</td>
<td></td>
<td>0/9</td>
</tr>
<tr>
<td>Prostate</td>
<td>0/9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TA = tubular adenoma; TVA = tubulovillous adenoma; IM = intestinal metaplasia; N.D. =
**Statistically significant expression in carcinomas compared with noninvolved tissues.

advanced gastric carcinomas (Kuniyasu et al. 1991, Saeki et al. 1994a). Interestingly, the incidence of CR-1 expression was found to correlate with the degree of dysplasia in the intestinal metaplasia and with tumor stage and patient prognosis in gastric cancer (Kuniyasu et al. 1994, Allgayer et al. 1997). In fact, the incidence of CR-1-positive cases was more frequent in late-stage, locally invasive tumors than in early-stage, noninvasive cancers. CR-1 expression in both groups was associated with a poorer patient prognosis. Finally, CR-1 expression has recently been reported in patients with long-term Helicobacter pylori infection, a risk factor for gastric carcinogenesis (Haruma et al. 2000). Collectively, these studies suggest that CR-1 overexpression in gastric premalignant lesions such as in the advanced stage of gastric cancer may be essential for the development and progression of this neoplasia.

Pancreatic cancer

CR-1 mRNA and protein have been detected by Northern blot analysis and immunohistochemistry in normal pancreas and in chronic pancreatitis, and are overexpressed in 43% of pancreatic ductal adenocarcinomas (Friess et al. 1994, Tsutsumi et al. 1994). The presence of CR-1 in pancreatic cancer cells has been found to correlate with advanced tumor stage, but not with the tumor grade or with the post-operative survival period of the cancer patients.

Colon cancer

CR-1 mRNA and protein are expressed at high levels in 73% of primary and metastatic colorectal cancers. CR-1 mRNA expression by Northern blot analysis was detected in 68% of primary or metastatic human colorectal cancers but only in 3% of noninvolved adjacent colon mucosa (Ciardiello et al. 1991b). Elevated CR-1 protein expression was also observed using immunohistochemistry in 79% of colon tumors, in 57% of tubulovillous or tubular adenomas and only in 12% of the noninvolved normal colonic mucosa adjacent to tumors or adenomas (Saeki et al. 1992). In another study, CR-1 expression was analyzed by immunocytochemistry in 41 colorectal carcinomas, 57 adenomas, 9 hyperplastic polyps and 98 normal mucosa samples (Saeki et al. 1995). No staining for CR-1 was observed in normal mucosa, whereas CR-1 expression was detected with an increasing proportion in 22% of hyperplastic polyps, 42% of adenomas and 78%
an autocrine growth regulation in the cancer cells may exist. Such as TGF α and AR or with the degree of dysplasia, and with the size and the histological subtype of colon adenomas (Saeki et al. 1994b). Nonpolypoid flat adenomas had a higher frequency of CR-1 expression than polypoid lesions. The gradual increase in CR-1 expression that is observed in the multistage process that evolves from adenoma to carcinoma suggests that CR-1 may be a tumor marker for human colorectal cancer and may be involved in the early events of colon carcinogenesis (Saeki et al. 1995). To support this hypothesis, a recent study has detected CR-1 expression in 62% of high risk normal colon mucosa specimens from individuals that belong to families with a high incidence of colorectal carcinomas, whereas only 20% of colon mucosa from low risk patients specimens were positive for CR-1 (De Angelis et al. 1999).

Rectal cancer
CR-1 immunoreactivity has been detected in 71% of primary rectal carcinomas and in 37% of normal rectal mucosa adjacent to carcinoma (Gagliardi et al. 1994). Interestingly, tumors with CR-1 immunoreactivity extending to the adjacent normal mucosa showed an increase in the incidence of bowel wall penetration, lymph node involvement and a recurrence rate of 100%, suggesting a correlation between CR-1 immunoreactivity in the normal adjacent colon mucosa and prognosis and survival of rectal tumors.

Gall bladder
CR-1 expression has been detected in 90% of gall bladder carcinomas without any correlation with tumor stage or patient prognosis (Fujii et al. 1996).

Expression of CR-1 in reproductive tract malignancies

Breast cancer
CR-1 mRNA and protein can be detected in several human breast cancer cell lines and in approximately 80% of human primary infiltrating breast carcinomas, in 47% of ductal carcinoma in situ (DCIS) and in only 13% of noninvolved adjacent breast tissue samples (Normanno et al. 1993, 1995, Qi et al. 1994, Panico et al. 1996). However, no significant correlations have been observed between CR-1 mRNA expression or immunoreactivity and various clinico-pathological parameters such as tumor stage, estrogen receptor status, lymph node involvement, histologic grade, proliferative index as assessed by Ki-67 staining or flow cytometry, LOH on chromosome 17p or overall patient survival (Dublin et al. 1995). CR-1 is frequently coexpressed with other EGF-related peptides in human breast cancer cells, such as TGFR, AR and HRGR, suggesting the possibility that an autocrine growth regulation in the cancer cells may exist.

Finally, a recent study has reported a positive correlation between nuclear erb B-4 expression and CR-1 expression in primary human breast carcinomas (Srinivasan et al. 2000). This finding is intriguing since it has been shown that CR-1 can indirectly enhance the tyrosine phosphorylation of erb B-4 through an unknown receptor (Bianco et al. 1999).

Endometrial cancer
CR-1 mRNA has been detected in normal human endometrium and in 19 endometrial tumor samples (Pfeiffer et al. 1997). CR-1 expression was higher in the endometrial carcinomas compared with normal endometrium but no correlation was observed with tumor stage and tumor differentiation. In another study, immunoreactivity for CR-1 was demonstrated in 67% of normal endometrium samples, in 30% of hyperplastic endometrium and in 65% of tumor samples (Niikura et al. 1995). In this study, CR-1 immunoreactivity in the tumor samples was correlated with surgical stage and progression of the disease. In addition, coexpression of CR-1 and EGFR, and CR-1 and TGFR, and not their single expression, was significantly associated with surgical stage, depth of myometrial invasion and peritoneal washing cytology. Finally, strong CR-1 immunoreactivity has been detected in endometrial atypical hyperplasia, an early event in endometrial carcinogenesis (Ayhan et al. 1998).

Cervical cancer
CR-1 has been detected using Western blot analysis in 38% of primary human keratinocytes, in 94.1% of HPV-16 immortalized keratinocytes and in 84.6% of cervical carcinoma cell lines (A D Ebert, J Stieler, M Nees, C Wechselberger, S Steinberg, C Woodworth, C Bianco, W Gullick, G Schaller & D S Salomon, unpublished data). CR-1 is also overexpressed in human cervical cancer samples compared with normal or premalignant samples. No statistically significant correlations were found between CR-1 expression and FIGO (International Federation of Gynecology and Obstetrics) stage, grading, lymph node involvement or patient age. However, Ertoy et al. (2000) recently found that CR-1 was overexpressed in a subpopulation of 26% of cervical carcinomas and was correlated with tumor size, lymphovascular space involvement, endometrial and parametrical involvement, and lymph node metastasis.

Ovarian cancer
CR-1 was initially detected by immunohistochemistry in 52% of ovarian adenocarcinomas (90% mucinous of high malignant potential and 50% serous), in 33% of mucinous tumors of low malignant potential and in 35% of cystadenomas (Niikura et al. 1997). In addition, a significant correlation was observed with advanced tumor stage when CR-1 was coexpressed with TGFR and AR or with the
EGFR, suggesting that coexpression of these EGF-related growth factors and the EGFR in a single tumor represents a growth advantage for the cancer cells through potential autocrine or paracrine mechanisms. In a more recent study, a large number of human ovarian carcinomas of different histologic types have been analyzed by immunohistochemistry for the expression of CR-1, TGFα and AR (A D’Antonio, S Losito, S Pignata, M Grassi, F Perrone, A De Luca, G Botti, W J Gulllick, G R Johnson, D S Salomon & N Normanno, unpublished data). Immunoreactive CR-1 was detected in 46% of ovarian carcinomas (83% mucinous and 53% serous), whereas no expression of CR-1 was found in endometrioid tumors. In addition, CR-1 expression did not correlate with lymph node involvement, presence of calcification or necrosis in the tumor, or number of mitoses. Interestingly, a significant increase in CR-1 expression was observed in tumor specimens obtained after chemotherapy when compared with tumors obtained from the same patient before the first surgery. Finally, the expression of CR-1 was significantly associated with short progression-free survival (PFS) and overall survival (OS) in a univariate analysis. However, this correlation was not observed in a multivariate analysis.

Expression of CR-1 in other cancers

CR-1 has been detected by immunohistochemistry in 60% of benign and malignant bladder tumors but no correlation between expression of CR-1 and tumor stage, tumor grade or clinical outcome has been found (Byrne et al. 1998).

In a large group of 195 stage I-IIIA non-small-cell lung carcinomas, CR-1 was found to be expressed at high levels in 91% of the adenocarcinomas (Fontanini et al. 1998).

In testicular carcinomas, CR-1 expression has been detected by Northern blot analysis and by immunostaining in all of the non-seminomatous tumors such as embryonal carcinomas and malignant undifferentiated teratocarcinomas that have been surveyed and in only 33% of the seminomas (Baldassarre et al. 1997). The preferential association of CR-1 expression with non-seminomatous tumors, that are more clinically aggressive, suggests a correlation between CR-1 and a more malignant phenotype in germ cell tumors.

Cripto-1 as target for therapy in human cancer

Since CR-1 is expressed at high levels in several different types of human malignancies, it may represent a useful target for experimental therapies in human cancer patients. Therapeutic antisense approaches have been successfully used to inhibit the growth of several human cancer cell lines in vitro and their growth as xenografts in vivo in nude mice (Neckers et al. 1992). Sequence specific antisense (AS) oligonucleotides or AS expression vectors can block the expression of specific proteins by binding to the corresponding mRNA and preventing translation. This approach has also been successfully utilized to impair CR-1 expression in several different types of human carcinoma cells. Inhibition of CR-1 expression in human GEO and CBS colon cancer cells by using either an amphotropic recombinant CR-1 AS mRNA retroviral expression vector or CR-1 AS phosphorothioate oligonucleotides results in a significant growth inhibition in vitro (Ciardiello et al. 1994). More importantly, GEO cells that were infected with the CR-1 AS retroviral vector exhibited a reduced tumorigenicity in nude mice in which tumors were smaller and appeared after a longer latency period as compared with noninfected GEO cells. Similar results were obtained in NTERA2 human embryonal carcinoma cells. Treatment of NTERA2 cells with 3 different CR-1 AS oligonucleotides or transfection with a CR-1 AS mRNA expression vector resulted in an inhibition of monolayer and soft agar growth in vitro (Baldassarre et al. 1996). Interestingly, the inhibition in CR-1 expression that was achieved in NTERA2 cells resulted in a significant reduction in the expression of a differentiation marker on these tumor cells. In another study, a superadditive effect was observed on reducing the growth of GEO cells in vitro when a CR-1 AS oligonucleotide was combined with a TGFα AS or AR AS oligonucleotide, suggesting that a combination of different growth factors is contributing to regulating the proliferation of colon cancer cells (Normanno et al. 1996). In fact, the anchorage-independent growth (AIG) of GEO cells was inhibited by 50% by each of the AS oligonucleotides at 5 µM but not with a scrambled missense oligonucleotide. However, the combination of the three AS oligonucleotides each at 1 µM resulted in 60% inhibition of the AIG of the GEO cells. Similarly, an additive growth inhibitory effect was observed in MDA-MB-468 human breast cancer cells and in several different human ovarian carcinoma cell lines that had been treated with a combination of a CR-1 AS oligonucleotide and either a TGF AS or AR AS oligonucleotide (De Luca et al. 1999, Casamassimi et al. 2000). Provocative data have also been obtained using combinations of CR-1 AS oligonucleotides with conventional chemotherapeutic drugs in colon cancer cells (De Luca et al. 1997). In a clonogenic assay, pretreatment of GEO cells with different concentrations of 5-fluorouracil, adriamycin, mitomycin C or cis-platinum induced an additive growth inhibitory effect when the cells were subsequently treated with a CR-1 AS oligonucleotide. More recently, CR-1 AS oligonucleotides have been used in combination with agents that block different but related intracellular signal transduction pathways (Normanno et al. 1999). A novel mixed backbone CR-1 AS oligonucleotide has been used in combination with a humanized anti-human epidermal growth factor receptor antibody MAb C225 and with 8-Cl-cAMP, a specific analog that inhibits type I protein kinase A. Low doses of each agent produced only a 15–35% growth...
inhibition in GEO cells in vitro but when the CR AS oligonucleotide was combined with either the MAB C225 or with 8-Cl-cAMP a synergistic antiproliferative effect occurred. Moreover, when the three agents were added together, a nearly complete suppression in the ability of GEO cells to grow in soft agar occurred. Finally, treatment with all three compounds induced apoptosis in GEO cells whereas single treatment or a combination of only two agents failed to induce any apoptosis.

**Future perspectives**

The recent identification of the EGF-CFC proteins as a new family of embryonic morphogens and as potential growth or differentiation factors in mammary epithelial cells raises a large number of important mechanistic questions. For example, the EGF-CFC proteins oep, Cr-1 and FRL-1 in zebrafish have been demonstrated through genetic analysis to act as competence or co-factors for nodal signaling during gastrulation while oep and cryptic function in a similar role during L/R axis formation (Ciccocigola et al. 1989, Dono et al. 1993, Kinoshita et al. 1995, Shen et al. 1997, Ding et al. 1998, Zhang et al. 1998, Gritsman et al. 1999, Xu et al. 1999, Yan et al. 1999, Gritsman et al. 2000, Schier & Shen 2000). Since nodal but not activin requires oep function and since both nodal and activin presumably activate the same set of activin type II and type I B receptors, it is not entirely clear as to the nature of the protein interactions that control these processes since direct binding of nodal or activin to oep or to other EGF-CFC proteins has not formally been demonstrated (Gritsman et al. 1999, Schier & Shen 2000). It is possible that oep and other EGF-CFC proteins might be functioning as co-receptors for nodal but not for activin by facilitating the presentation of nodal to an activin type IIB receptor in a manner analogous to the glial cell-derived neurotrophic factor (GDNF)/c-ret tyrosine kinase receptor signaling system (van Weering & Bos 1998, Saarma & Sariola 1999, Trupp et al. 1999). In this system, GDNF and other proteins such as artemin, neuturn and persephin that are distantly related to TGF-β do not bind directly to c-ret but bind to a family of four GPI-anchored co-receptors, GDNF family receptor α1–4 (GFRα) (Saarma & Sariola 1999, Trupp et al. 1999). Ligand-bound GFRα proteins then utilize either c-ret or in some cases c-src as signaling components in a tertiary complex. Two additional examples are also noteworthy and illustrate the fact that proteins may be multifunctional and capable of interacting with multiple signaling pathways. Neurilpin-1 (NRP-1) is a transmembrane glycoprotein that is a receptor for several members of the semaphorin/collapsin family that are involved in neuronal guidance (Miao et al. 1999, Gagnon et al. 2000, Raper 2000). NRP-1 is also a co-receptor for a specific isoform of vascular endothelial growth factor (VEGF) VEGF_{165}, but not for VEGF_{121}, which presents VEGF_{165} to the VEGF tyrosine kinase receptor-2, Flk-1/KDR (kinase insert-domain-containing receptor) (Miao et al. 1999, Gagnon et al. 2000). In addition to NRP-1, VEGF_{165} but not VEGF_{121} also binds to glypican-1, a cell-associated protein that is GPI-anchored to the membrane (Gengrinovitch et al. 1999). Glypican-1 potentiates the binding of VEGF_{165} to the VEGF receptor Flk-1/KDR. Likewise, dally is a heparin-sulphate containing proteoglycan in Drosophila that resembles a glypican (Jackson et al. 1997, Lin & Perrimon 1999, Tsuda et al. 1999). Dally can function as a co-receptor for both Wingless (Wg)/wnt-1 and a TGFβ-related protein, decapentaplegic (dpp), presenting these proteins to either the wnt receptor, frizzled or to a TGFβ receptor (Jackson et al. 1997, Lin & Perrimon 1999).

EGF-CFC proteins might also serve as antagonists for a signaling pathway(s). For example, cerberus is a multifunctional antagonist that can bind and neutralize nodal, BMP-4 and wnt-1 (Piccolo et al. 1999, Belo et al. 2000). In addition, tomoregulin, a peptide that specifically binds to the type 1 erb B-4 tyrosine kinase receptor with low affinity, also possesses two follistatin-related domains that have the potential to bind and neutralize activin (Uchida et al. 1999). Therefore, oep or other EGF-CFC proteins might function by binding to and subsequently neutralizing an antagonistic, nodal-specific binding protein, thereby permitting nodal to activate an activin type IIB-type IB receptor-Smad-2 signaling pathway. In this context, it has recently been demonstrated that overexpression of either a wild-type or a secreted, COOH-terminal truncated form of oep can antagonize either BMP or nodal signaling in zebrafish and Xenopus (Kiecker et al. 2000). Finally, oep or EGF-CFC proteins might have the capacity to co-activate a parallel signaling pathway after binding to nodal that is necessary, in conjunction with the activin type II and type I B receptor-Smad-2 signaling pathway, for eliciting a biological response. This co-stimulatory pathway may depend, in part, upon activation of downstream MAPK by an upstream tyrosine kinase(s) (e.g. erb B-4, FGFR-1 or src-like kinase) (LaBonne & Whitman 1984, Weinstein et al. 1998). In this respect, TGFβ1 has been shown to activate the rasraf/MAPK pathway which in epithelial cells is obligatory for eliciting some TGF responses (Hartsough & Mulder 1997, Mulder 2000). These latter two pathways may not be mutually exclusive in a mechanistic framework since there is experimental evidence demonstrating that the cloned and purified mammalian TGFβRII receptor not only has a serine-threonine kinase activity but also an associated tyrosine kinase activity that is not directly activated by TGFβ1 (Lawler et al. 1997). In fact, there is strong evidence in zebrafish, Xenopus and mouse that co-activation of a FGFR-1 signaling pathway is also necessary to complete gastrulation fully and for L/R asymmetry development (Kimelman et al. 1992, Beddington & Smith 1993, Cornell & Kimelman 1994, Harland & Gerhart 1997, Hartsough &
The mechanism by which activin, nodal and fgf-8 cooperate in these processes is not completely defined. However, it is intriguing that both human CR-1 and Xenopus FRL-1 can in some manner indirectly transactivate FGFR-1 (Kinoshita et al. 1995, C Bianco, C Wechselberger & D S Salomon, unpublished data). Likewise, the ability of CR-1 specifically to transactivate erb B-4 may also be of developmental significance since HRGβ1, a ligand for erb B-4, is expressed in the gastrula of Xenopus and can induce mesoderm formation in animal cap explants in vitro (Chung & Chung 1999). In addition, HRGβ1 and erb B-4, like Cr-1, are important determinants in early cardiogenesis (Dono et al. 1993, Xu et al. 1998, 1999, Klapper et al. 2000). These examples merely serve to illustrate the potential complexity by which the EGF-CFC proteins might function as either co-receptors for one or several distinct groups of structurally unrelated proteins and receptor signaling pathways and/or as antagonistic binding proteins or modulators for multiple ligands. The expression and purification of a biologically active form of an EGF-CFC protein will be useful for the identification of a ligand(s) and/or receptor for this family of proteins and its potential coupling to other signal transduction pathways.

Irrespective of the molecular mechanism by which EGF-CFC proteins might function, the diversity of biological effects that can be induced by these proteins is large and similar to the pleiotropic spectrum of responses induced by other growth factor families. In this respect, the ability of these proteins to induce cell motility in embryonic epiblast cells of the primitive streak, in mouse mammary epithelial cells and in human carcinoma cells is noteworthy (Xu et al. 1998, 1999, Ebert et al. 2000, C Wechselberger, A D Ebert, C Bianco, B Wallace-Jones, N Khan, Y Sun & D S Salomon, unpublished data). In all cases, this is preceded by an EMT since cells assume a more mesenchymal phenotype. In the case of some of the epithelial cells, there is a loss of E-cadherin function and an up-regulation in vimentin expression during this conversion (Ebert et al. 2000, C Wechselberger, A D Ebert, C Bianco, B Wallace-Jones, N Khan, Y Sun & D S Salomon, unpublished data). Loss of E-cadherin function may be particularly relevant to the case of some of the epithelial cells, in type I collagen gels, and in vivo in the cleared mammary fat pad in Cr-1-transduced cells (Crawford et al. 1999). Additionally, these cellular alterations may contribute to the increased migratory and invasive behavior of tumor cells overexpressing cripto. It is more than likely that other growth factors such as nodal, activin, TGFβ1 and/or FGF may be obligatory for or accentuate these biological responses to different members of the EGF-CFC family. These biological effects may be functionally important since cripto is an oncofetal protein. In this respect, mouse Cr-1 and human CR-1 are differentially overexpressed in a number of different types of carcinomas and in premalignant lesions from these same tissues. Identification of factors activating transcription factors that can bind to the promoter regions of these genes will therefore be important in determining the mechanism by which the expression of these genes are up-regulated in tumor cells. Reciprocally, identification of downstream genes that might be regulated by EGF-CFC proteins will be useful in ascertaining the mechanism by which EGF-CFC proteins function.

Development of animal models in which mouse or human cripto genes are transcriptionally regulated by tissue-specific promoters will be necessary to determine if misexpression and/or overexpression of these genes can contribute to abnormal tissue development and neoplasia. For example, the use of the WAP or MMTV promoters for directing expression of cripto to the epithelial cells of the mammary gland in mice will also be useful in assessing the in vivo effects of cripto overexpression at various stages of mammary gland development and involution. Studies to generate such mice are currently in progress. The observation that Cr-1 expression is elevated during pregnancy and lactation in the mouse and that exogenous CR-1 protein can inhibit β-casein and WAP expression in response to lactogenic hormones in vitro suggests that ectopic expression of this gene is likely to produce a mammary phenotype and possibly perturb normal mammary differentiation and development (DeSantis et al. 1997, Niemeyer et al. 1998). In addition, the development of appropriate transgenic mice in which cripto expression is directed to the mammary gland will facilitate assessment of the potential function that cripto might perform at various stages in mammary epithelial cell transformation and tumor progression. The development of ductal hyperplasias in the cleared mammary fat pad of virgin mice that harbor Cr-1-transduced mammary epithelial cells portends such a possibility (Kenney et al. 1997b, Niemeyer et al. 1999). Finally, Cr-1 can potentially be ablated in the mammary gland by crossing WAP-Cre mice with mice that have had the endogenous Cr-1 gene flanked by two loxP sites. F1-hybrid mice that contain the WAP-Cre gene and the floxed Cr-1 gene should possess cells expressing the Cre recombinase gene under the transcriptional control of the WAP promoter which should then lead to the selective excision and inactivation of the Cr-1 gene in the mammary gland during pregnancy where the WAP promoter is normally expressed (Wagner et al. 1997). This approach will be necessary to study the role of the Cr-1 gene in postnatal mammary gland development since homozygous, germline deletion of the Cr-1 gene in the mouse is embryonically lethal.
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