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UMI
CALRETICULIN MODULATES CELL ADHESIVENESS

by

Greta Kaur Jass

Thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Anatomy and Cell Biology
University of Toronto

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ABSTRACT

Calreticulin (CRT) is a multifunctional, ubiquitously expressed Ca²⁺ binding protein of the endoplasmic reticulin (ER). The ability of CRT to modulate cellular adhesion has created some controversy as to where, subcellularly the CRT protein functions in this capacity. I will show that the full length ER form of CRT is involved in cellular adhesion rather than a cytosolic form of CRT, as proposed by others. Overexpression of CRT results in increased cell adhesion which is visualized by increased cell spreading, actin stress fiber formation, fibronectin matrix deposition, reduced cell motility, and epithelial-like-monolayer migration. CRT overexpression coordinately increases expression of cytoskeletal proteins vinculin and β-catenin, and the cell surface protein N-cadherin, which can explain all the effects of increased adhesion. I propose that the effects of differential CRT expression are modulated by the ER localized CRT, and that this may be realized via ER-to-nuclear signal transduction.
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<thead>
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<th>Description</th>
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<tbody>
<tr>
<td>BAK</td>
<td>L fibroblast stable transfectants underexpressing CRT</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>calcium ion</td>
</tr>
<tr>
<td>[Ca²⁺]</td>
<td>calcium ion concentration</td>
</tr>
<tr>
<td>cDNA</td>
<td>recombinant deoxyribonucleic acid</td>
</tr>
<tr>
<td>CRT</td>
<td>calrectulin</td>
</tr>
<tr>
<td>C-DMEM</td>
<td>carboxyl terminal Dulbecco's modified essential medium</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>ES</td>
<td>embryonic stem cells</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GFKR</td>
<td>glycine-phenylalanine-phenylalanine-lysine-arginine</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GRP</td>
<td>glucose response protein</td>
</tr>
<tr>
<td>HA</td>
<td>haemagglutinin</td>
</tr>
<tr>
<td>HG</td>
<td>high glucose</td>
</tr>
<tr>
<td>Hr/s</td>
<td>hour/s</td>
</tr>
<tr>
<td>IP₃</td>
<td>inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>IRM</td>
<td>interference reflection microscopy</td>
</tr>
<tr>
<td>KAB</td>
<td>L fibroblast stable transfectants overexpressing CRT</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>KDEL</td>
<td>lysine-aspartate-glutamate-leucine</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
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<td>µm</td>
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<tr>
<td>µM</td>
<td>micromolar</td>
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<tr>
<td>mM</td>
<td>millimolar</td>
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<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>MW</td>
<td>molecular weight</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>nm</td>
<td>nanometer</td>
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<tr>
<td>nM</td>
<td>nanomolar</td>
</tr>
<tr>
<td>P-</td>
<td>proline-rich</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PDI</td>
<td>protein disulfide isomerase</td>
</tr>
<tr>
<td>PGK</td>
<td>L fibroblast stable mock transfectants</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>pp</td>
<td>phosphoprotein</td>
</tr>
<tr>
<td>RGD</td>
<td>arginine-glycine-aspartate</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarcoplasmic-endoplasmic calcium ATPases</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>TRIS</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>Zn^{2+}</td>
<td>zinc ion</td>
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Calreticulin (CRT) is a major Ca\textsuperscript{2+}-binding protein of the endoplasmic reticulum (ER) of non-muscle cells. In addition, recent evidence suggests that CRT is a multifunctional protein as it is a chaperone and it modulates gene expression. Most importantly, ample evidence has accumulated showing that an ER-resident CRT affects cell adhesion. Based on this conjecture several mechanisms of CRT action can be envisaged: two major ones include a direct protein-protein interactions between CRT and \(\alpha\)-integrin, which are a major constituent of strong cell attachments, focal contacts; while the second one is based on a hypothesis that CRT plays a role in ER-to-nucleus "communication".

Previous adhesion assays conducted with mouse L fibroblast stable transfectants expressing differential levels of the CRT protein show coordinate changes of integrin specific cell attachment, spreading and shape. In this study I will use additional adhesion assays to examine this correlation between CRT expression and the degree of cell adhesiveness. These assays will be studying changes in cell spreading and strength of attachment via actin stress fiber formation, deposition if fibronectin matrix meshwork, and the measurement of cellular velocity. Both differences in cell-substratum and cell-cell interactions will be studied by immunofluorescence detection of cytoskeletal adherens junction protein, vinculin, which is localized to both cell-cell and cell-substratum junctions. A wound healing experiment will be used to observe differences
As CRT was originally hypothesized to affect cellular adhesion by directly binding to α-integrins in focal adhesions, localization of CRT will be extensively examined. Immunofluorescence double labelling of CRT and vinculin will be done to see whether or not CRT is detectable in focal adhesions. Integrins will be artificially clustered on the cell surface to recreate the experimental conditions in which the only reported in vivo co-localization between CRT and integrins was achieved. Lastly, as CRT must be present in the cytosol for direct protein-protein interactions with α-integrins cell lines expressing green fluorescent protein (GFP)-tagged CRT targeted to both the ER and cytosol will be utilized to observe the localization of CRT with respect to focal adhesions. Therefore, the objective of the proposed research is to study the phenomenology of effects of CRT on the cell adhesion with particular attention paid to the question of whether or not CRT is detectable in focal contacts.
Calreticulin (CRT) is a ubiquitous, eukaryotic protein. It is one of the major calcium-binding proteins of the endoplasmic reticulum (ER) as depicted by its name (Calcium/reticulum) (Michalak et al., 1992).

CRT was first isolated from rabbit skeletal muscle sarcoplasmic reticulum (SR) as a Ca\(^{2+}\)-binding, water soluble, acidic protein (Ostwald and MacLennan, 1974). Subsequently, Khanna and Waisman (1986) analysed different bovine tissues for the presence of CRT and showed that all tissues tested, with the exception of erythrocytes, contained CRT. Higher amounts of CRT were found in the pancreas, liver, and testis, while relatively low amounts of CRT were found in the cerebral cortex, and in smooth, skeletal, and cardiac muscle. These results are in agreement with latter studies of CRT distribution in rabbit tissues (Tharin et al, 1992). CRT cDNA has been isolated from cDNA libraries from all organisms studied to date (Michalak et al, 1992; Nash et al, 1994), with the exception of yeast where neither the presence of CRT gene, mRNA nor protein has been found (Krause and Michalak, 1997). Analysis of the cDNA sequence from human, mouse, rabbit, rat, Aplysia, Xenopus, Drosophila, C.elegans and S.mansonii reveals CRT to be a highly conserved protein. There is 90% sequence homology between CRT isoforms from human, rabbit, mouse and rat (Michalak et al, 1992). CRT has also been identified in plant cells, for example, Tradescantia and sugar beet protoplasts, and barley (Opas et al., 1996b). Plant CRT is similar to CRT of mammalian
in human and mouse was found to exist as a single copy on the short arm of chromosome 19 (McCauliffe et al, 1992). To date there is no evidence of splicing of transcribed mRNA encoding CRT (Krause and Michalak, 1997).

**CRT STRUCTURE**

CRT cDNA was isolated and sequenced from rabbit skeletal muscle SR (Fliegel et al, 1989). The cDNA encodes 418 amino acids, of which a 17-residue NH$_2$-terminal signal sequence is cleaved during synthesis. This signal sequence is responsible for the targeting of CRT to the ER. Although the predicted molecular weight of CRT is 46,567 Da the protein migrates at a higher molecular weight (MW) on a SDS-PAGE gel, thus CRT is generally known as a 60 kDa protein.

The structure of CRT has been extensively reviewed (Fliegel et al, 1989; Michalak et al. 1992; Nash et al, 1994). The predicted structure of CRT based on its amino acid sequence is depicted in Figure 1. CRT can be divided into three domains: 1) N-domain, the amino-terminal which is a globular domain consisting of an extreme amino terminal helix-turn-helix motif followed by eight anti-parallel $\beta$-sheets; 2) P-domain, is called the proline-rich region since it contains the three 17-amino-acid repeats (PxxIxDPDaXKPEDWDE) which are followed by a proline, serine, and threonine rich segment, and a zinc binding region; and 3) C-domain, carboxyl-terminus, which is a
highly acidic domain, of the last 37 amino acid residues 37 are aspartic or glutamic acid, and terminates with the ER retention sequence, KDEL.

![Diagram of CRT structure]

Fig. 1. Proposed structural model of CRT (modified with permission. M. Opas, 1991).

CRT contains two Ca\textsuperscript{2+} binding domains and one Zn\textsuperscript{2+} binding domain. A high affinity and low capacity Ca\textsuperscript{2+} binding region (binds 1 mole of Ca\textsuperscript{2+} per 1 mole of CRT, \(K_d = \sim 1\mu M\)) is located in the P-domain, while a low affinity and high capacity binding region (binds 25 moles of Ca\textsuperscript{2+} per 1 mole of CRT, \(K_d = \sim 250\mu M\)) is located within the C-domain (Ostwald and MacLennan, 1974; Fliegel et al, 1989; Michalak et al, 1992;
The Zn\(^{2+}\) binding domain is localized to the P-domain and found to bind 18 moles of Zn\(^{2+}\) per 1 mole of CRT, \(K_d = \sim 300\mu M\) (Baksh et al, 1995b).

The C-domain contains a putative glycosylation site, but only bovine (Fliegel et al, 1989) and rat (Peter et al, 1992) liver CRT have been found to be glycosylated. CRT also has a putative nuclear localization signal sequence, as well as, putative recognition sequences for protein kinase C, casein kinase II and tyrosine kinase phosphorylation; all of which have been deduced by the analysis of the amino acid sequence of CRT. However, these have yet to be shown to be functional in vivo (Michalak et al, 1992; Nash et al, 1994).

The greatest divergence from CRT's highly conserved amino acid sequence is seen with *Onchocerca volvulus*, where the C-domain is basic rather than acidic as in mammalian CRT (Michalak et al, 1992). Also, a neuronal retinal isoform of CRT or an unknown protein with antigenicity similar to CRT was identified in rabbit retina, and was found to have a slightly higher MW than purified dog pancreatic CRT (Opas and Michalak, *et al*, 1992; Tharin *et al*, 1992).

**LOCALIZATION OF CRT**

The localization of CRT to the ER is well established (Michalak *et al*. 1992; Nash *et al*, 1994). This localization is ensured by the extreme N-terminal signal sequence and the C-terminal KDEL sequence. Retention of CRT in the ER has been studied by
oligosaccharides of mixed hybrid type with terminal galactoses. The enzyme required for this glycosylation, galactosyltransferase, is almost exclusively found in the trans-Golgi. Therefore, this isoform of CRT was shown by pulse-chase experiments to go to the trans-Golgi before being transported retrogradely to the ER without being able to escape into the surrounding medium. Moreover, Nycodenz-density gradient centrifugation was used to demonstrate that CRT mainly resides in the rough ER. Recently, studies of CRT retention show CRT to be retained in the ER in COS cells overexpressing CRT by 50-100 fold (Sönrichsen et al, 1994). The C-terminal domain of CRT is involved in retaining the protein within the ER so that only a small proportion is able to escape, which is then retrieved back to the ER by the KDEL sequence-dependent mechanism.

The positive identification of CRT as a Ca\(^{2+}\) sequestering protein found in rough pancreatic microsomes led some to believe that CRT may function from specialized and distinct organelles called calciosomes (Rossier and Putney, 1991). However, Michalak et al (1991) proved this localization of CRT to pancreatic microsomes or calciosomes to be artifactual. CRT was, thus, once again shown to be localized in the lumen of the ER of pancreatic cells.

CRT was also detected in the nuclear envelope in human skeletal muscle and in MDCK cells, and additionally in nucleoli-like structures in the L6 rat myogenic cells (Opas et al, 1991). The localization of CRT to nucleoli and nuclear envelope was also
contain CRT (Dedhar et al, 1994), where CRT has been proposed to modulate gene expression (Burns et al., 1994a; Dedhar et al., 1994) by interacting with the DNA-binding domains of glucocorticoid and androgen hormone receptors. Despite these reports, the localization of CRT in the nucleus is still questionable. Recently Michalak et al (1996) separated the outer nuclear membrane from sucrose-gradient purified nuclei and showed that CRT was only present in the outer nuclear membrane fraction and not detectable in the nuclear fraction. As the ER membrane is continuous with the outer nuclear membrane, removing the latter eliminates the possibility of nuclear fraction contamination by ER CRT. Previous reports of the localization of CRT in the nucleus (Opas et al, 1991) are now considered artifactual (Michalak et al, 1996).

Localization of CRT to lipid droplet was seen during lactation, where CRT was found to be associated with lipid droplets during the extreme conditions of lactation but not during pregnancy or involution (Ghosal et al, 1994). CRT was also detected in the acrosome of rat sperm (Nakamura et al, 1993) and the lysosome-like secretory granules of cytosolic T lymphocytes (Dupuis et al, 1993). In the latter case, CRT is thought to function as a Ca$^{2+}$ chelator, thus preventing organelle autolysis by inhibition of the cytolytic protein, perforin which becomes activated in the presence of Ca$^{2+}$.

Cellular adhesion has been proposed to be modulated by CRT localized at the plasma membrane both extracellularly (White et al, 1995) and intracellularly (Dedhar, 1994; Leung-Hagesteijin et al, 1994). Evidence for the presence of CRT these
has been demonstrated by Opas' group (1996a), that CRT which is detectable by immunofluorescence microscopy in mouse L fibroblast and rat A10 smooth muscle cells is confined to the ER.

**CRT EXPRESSION**

CRT is considered a non-muscle functional analogue of calsequestrin, a striated muscle Ca\(^{2+}\) storage protein (Michalak and Milner, 1991). Accordingly, Koyabu *et al*, (1994) has demonstrated that there is a switching from the expression of CRT to expression of calsequestrin during skeletal muscle differentiation in chick. In contrast, as L6 myogenic cells are induced to differentiate and form myotubes, CRT expression was found to remain constant while the expression of calsequestrin steadily increased (Tharin *et al*, 1996).

The expression of CRT was studied in response to both Ca\(^{2+}\) shock (by the addition of the Ca\(^{2+}\) ionophore A23187), heat shock, as well as, activation of protein kinase C by the tumour promoter, TPA (Opas *et al*, 1991). In all cases no significant increase in CRT expression was detected. However, recently it has been reported that CRT gene expression can be increased by treatment with either the Ca\(^{2+}\) ionophore, A23187, or the ER Ca\(^{2+}\)-ATPase inhibitor, thapsigargin, both in a cyclohexamide dependent manner (Wasem *et al*, 1997). CRT was also described as a heat shock/stress
response gene (Sauvage et al, 1993; Nguyen et al, 1996). Experiments in human epidermoid squamous carcinoma cell line induced transcription of CRT by heat shock, Ca$^{2+}$ ionophores, and heavy metals such as zinc and cadmium (Nguyen et al, 1996).

CRT AS A MULTIFUNCTIONAL PROTEIN

Although CRT was first described as a Ca$^{2+}$-binding protein, additional roles have been found for it over the last two decades. Here only the following four major functional aspects of CRT will be discussed: (I) CRT as a Ca$^{2+}$ binding protein; (II) CRT as a chaperone protein; (III) modulation of gene expression by CRT; and (IV) modulation of cell adhesiveness by CRT.

CRT as a Calcium-Binding Protein

Regulation of cytosolic Ca$^{2+}$ concentrations ([Ca$^{2+}$]) is very tightly controlled in order to maintain the 20,000 fold difference between intracellular Ca$^{2+}$ levels (~100nM) and extracellular Ca$^{2+}$ (~2mM) (Clapham, 1995). Ca$^{2+}$ is the most common signal transduction element in cells, and it is involved in the regulation of many basic cellular processes. CRT is one of the several Ca$^{2+}$-binding proteins in the ER lumen (Michalak and Milner, 1991; Milner et al, 1992; Nash et al, 1994; Tharin et al, 1992; 1996), and recently luminal ER proteins such as GRP94, GRP78, PDI, and calcistorin have been shown to bind Ca$^{2+}$ with relatively high capacity (Cala, et al, 1990; Milner et al, 1992,
As CRT, a Ca$^{2+}$ binding protein, was found to lack an EF-hand consensus Ca$^{2+}$-binding sequence, there was interest in localizing the high and low affinity Ca$^{2+}$-binding sites to the P-domain and C-domain of CRT (Baksh and Michalak, 1991). Recent reports have revealed the ability of CRT to modulate intracellular Ca$^{2+}$ storage. The groups of Pozzan (Bastianutto et al, 1995) and Krause (Mery et al, 1996), demonstrated that overexpression of CRT in cells by 1.6 and 3.5 fold resulted in a 2.1 and 2.2 fold increase of cellular Ca$^{2+}$ content, respectively. This increased abundance of Ca$^{2+}$ is believed to be stored in the ER lumen, as the stored Ca$^{2+}$ was almost completely depleted with thapsigargin treatment which inhibits sarcoplasmic-endoplasmic reticulum Ca$^{2+}$-ATPases (SERCA). SERCAs are the Ca$^{2+}$ pumps responsible for Ca$^{2+}$ uptake into the ER, while Ca$^{2+}$ release is effected via the inositol 1,4,5-trisphosphate (IP$_3$) receptor (Clapham, 1995). The contribution of CRT towards the thapsigargin-sensitive ER Ca$^{2+}$ storage/buffering capabilities is calculated to be approximately 47% (Bastianutto et al, 1995). An increase in Ca$^{2+}$ storage capacity of cells overexpressing CRT has been also shown to decrease store-operated Ca$^{2+}$ influxes (Bastianutto et al, 1995; Mery et al, 1996). Despite increases the abundance of the ER lumenal CRT and enhanced Ca$^{2+}$ storage, cytosolic [Ca$^{2+}$] remains unchanged under normal culture conditions (Bastianutto et al, 1995; Mery et al, 1996; Opas et al, 1996a). However, when L fibroblast cells are challenged with stepwise addition of ionomycin, stepwise influxes of Ca$^{2+}$ are induced in underexpressers of CRT, but are buffered down in CRT overexpressers (Opas et al, 1996a).
inhibited repetitive IP$_3$ induced Ca$^{2+}$ waves (Camacho and Lechleiter, 1995). Also, overexpression of CRT protects cells from growth inhibition and death in conditions of low extracellular [Ca$^{2+}$], L fibroblast underexpressers of CRT cultured in medium containing not more than 20mM Ca$^{2+}$ underwent drastic reduction of growth rate with only 57% viability, while CRT overexpressers demonstrated 90% viability and their growth rate was almost unimpaired under the same growth conditions (Opas et al, 1996a).

**CRT as a Chaperone Protein**

CRT as molecular chaperone binds to newly synthesized intermediates of monoglucosylated proteins (Helenius et al, 1997). These chaperone properties of CRT are similar to those of the chaperone protein, calnexin, which is a nonglycosylated type I membrane protein. CRT was shown to function as a chaperone in the biosynthesis of myeloperoxidase (MPO), a lysosomal heme protein found in neutrophils and monocytes, as a glycosylated, heme-free early precursor of MPO interacts transiently with CRT (Nauseef et al, 1995). Both CRT and calnexin were shown to interact in vitro with the glut 1 glucose transporter (Oliver et al, 1996) and influenza haemagglutinin (HA) (Peterson et al, 1995; Hebert et al, 1996). In the case of HA, calnexin bound to all folding intermediates while CRT only bound to an early oxidative form of HA, thus
CRT has also been shown to interact with protein disulfide isomerase (PDI), \textit{in vitro}, most likely in a zinc-dependent manner (Baksh \textit{et al}, 1995a). PDI catalyses the isomerization of intramolecular disulfide bridges, and folding of a variety of proteins (Noiva \textit{et al}, 1992). The N-domain of PDI binds strongly to the P-domain of CRT but only weakly to its N-domain (Baksh \textit{et al}, 1995a). These PDI-CRT interactions reportedly prevent high affinity Ca$^{2+}$ binding by CRT, while the N-domain of CRT inhibits PDI's ability to refold scrambled RNase A, \textit{in vitro} (Baksh \textit{et al}, 1995a).

Additionally, analysis of the promoter region of CRT and the glucose response proteins, GRP78, GRP94 and PDI reveal there are similar multiple CCAAT-like motifs as well as GC-rich regions (McCauliffe \textit{et al}, 1992). GRP78, GRP94 and PDI are all acidic, Ca$^{2+}$-binding chaperone proteins that localize to the ER. These similarities indicate that these proteins may have some common regulatory sequences, and thus be coordinately induced.

\textbf{Modulation of Gene Expression by CRT}

The ability of CRT to modulate gene expression by inhibiting steroid hormone receptors has been studied by two groups, that of Marek Michalak (Burns \textit{et al}, 1994a) and Shoukat Dedhar (1994). CRT binds \textit{in vitro} to the synthetic peptide KLGFFKR (Rojiani \textit{et al}, 1991), which is very similar to the highly conserved DNA-binding domain
been shown in vitro to bind the androgen receptor, thus preventing its binding to the $^{32}$P-labelled 26-base-pair DNA androgen-response element (Dedhar et al, 1994). Moreover, Burns et al (1994a) demonstrated that in vitro the N-domain of CRT is involved in directly binding to the glucocorticoid receptor. This, in turn, prevents it from binding to the glucocorticoid-response element. Overexpression of CRT in mouse L fibroblasts decreases the formation of protein products of the glucocorticoid-sensitive cytochrome P450 gene. Conversely an increase was seen in underexpressers of CRT. It was thought, that CRT to bind to steroid hormone receptors it must first be present in the cytosol in order for CRTs putative nuclear localization signal to target CRT to the nucleus (Burns et al, 1996b). Hence additional transfection studies were carried out with full length ER CRT and a truncated cytosolic form of CRT (CRT minus its leader signal sequence), which revealed that only full length CRT and not the truncated cytosolic form of CRT modulates glucocorticoid-sensitive gene expression (Michalak et al, 1996). Therefore, it is currently thought that CRT does not directly bind glucocorticoid or other steroid receptors in the cytosol in vivo, but somehow modulates their function from its localization in the ER.

Modulation of Cell Adhesiveness by CRT

Investigation of the role of CRT in cellular adhesion was initiated when CRT was
The rational for this experimentation was to determine which proteins bind the amino acid sequence KxGFFKR, which is the most conserved sequence of the cytoplasmic region of α-integrins, (Rojiani et al, 1991; Sastry and Horwitz, 1993; O'Toole et al, 1994; Williams et al, 1994). α-integrins are one of the transmembrane subunits of integrins that are, together with the β-integrin subunit, involved in formation of focal adhesions.

Focal adhesions or focal contacts are a type of cell-substratum adhesion, a subclass of adherens-type junctions. They are strong adhesions where the undersurface of the cell comes closest to the substratum. There is only 10-15nm separating the cell from the substratum, and focal adhesions are generally 2-10μm long and 0.25-0.5μm wide (Burridge et al, 1988). Focal adhesions are important in many cellular processes (Williams et al, 1994). A variety of diseases involve regulation of adhesion, for example: leukocyte chemotaxis, angiogenesis, epithelial barrier disfunction and tumour metastasis (Sjaastad and Nelson, 1997). Focal adhesions have been extensively reviewed (Burridge et al, 1988; Jockusch et al, 1995; Burridge and Chrzanowska-Wodnicka, 1996), and are composed of transmembrane proteins which links the cell from the extracellular matrix (ECM) to an intracellular adhesion plaque of proteins which connects actin stress fibers. The most studied transmembrane linker of focal adhesions is the heterodimeric superfamily of integrins, which consists of α- and β-integrin subunits. Many isoforms of both α- and β-integrins exist, all containing one
transmembrane-spanning domain, a large extracellular glycosylated N-domain and a short intracellular C-domain. At least 20 combinations of integrin heterodimers have been identified (Sjaastad and Nelson, 1997), but there is a functional redundancy as a heterodimer can recognize several ECM proteins and an ECM protein can be recognized by several heterodimers (Jockusch et al, 1995). It is mainly α-integrin which is able to recognize and bind to ECM proteins such as fibronectin, vitronectin, collagen, laminin, fibrinogen and thrombospondin. Integrins must first be clustered before focal adhesions can be assembled with intact cytoplasmic adhesion plaques and actin stress fibers (Burridge and Chrzanowska-Wodnicka, 1996). Proteins found in association with cytoplasmic plaques can be categorized into two groups: 1) structural proteins, which link actin stress fibers, such as actin, talin, vinculin, α-actinin, filamin, tenein, radixin, and tensin/insertin; and 2) regulatory proteins, including FAK, paxillin, zyxin, profilin, gelsolin, proteases, phospholipases, protein kinases, serine/threonine kinases, myristoylated alanine-rich C-kinase substrate, proline motif proteins, and GTP-binding proteins (Jockusch et al, 1995). Talin and paxillin are the only two proteins of those mentioned above that are thought to be exclusively localized to focal adhesion. Despite the vast investigation of focal adhesions their exact mechanisms of formation, regulation, function, and interactions of associated proteins remains to be resolved.

The ability of CRT to modulate cell adhesiveness has been centred around the in vitro interactions between CRT and the cytoplasmic domain of α-integrin subunit. Mutant α-integrin subunit transfection studies revealed that this domain plays an
between the β-integrin subunit cytoplasmic domain and other adhesion molecules (Yläne et al, 1993). More specifically, disruption of the α-integrin consensus sequence GFFKR resulted in a switch from a default low affinity binding state to a high affinity binding state of integrin heterodimers (O'Toole et al, 1994). Therefore, the GFFKR motif is believed to be involved in the regulation of the ligand affinity of integrin heterodimers. In an attempt to co-localize CRT to focal adhesions or integrin clusters, an antibody cross-linking procedure was used to induce integrin clusters on the cell surface of PC-3 human prostate carcinoma cells, followed by immunofluorescence co-localization of CRT (Leung-Hagesteijn et al, 1994). Also, stimulation of Jurkat T-lymphoblastoid cells with anti-integrin antibodies or phorbol 12-myristate 13-acetate (PMA) induced cell adhesion and increased CRT co-immunoprecipitation with integrin (Coppolino et al, 1995), which was taken as an indication that there is direct binding in vivo between CRT and the α-integrin subunit as cells adhere to their substratum. Stably transfected L fibroblast cell lines expressing elevated or reduced levels of CRT were used along with mock-transfectants to examine the effects of varying levels of CRT expression on cell attachment, morphology and growth (Opas et al, 1996a). CRT overexpressers attached more avidly to the ECM protein coated substrata than either control cell lines or CRT underexpressers; this effect was most pronounced on fibronectin. The most obvious morphological changes associated with alterations in the cell area and the number and length of filopodia. Quantitative morphometric analysis
The cell shape was measured as a divergence of the cell shape from a circle. The results of these studies indicate there is a direct correlation between CRT expression and cell area and an inverse relationship between CRT expression and the cell “roundness” in these transfectants (Opas et al, 1996a). Severely impaired integrin-mediated adhesion is seen in both CRT-deficient embryonic stem (ES) cells and embryonic fibroblasts isolated from CRT mutant mice, although there is no change in the level of integrin expression (Coppolino et al, 1997). Moreover, transfection of these cells with recombinant CRT rescues cell adhesiveness.

On the other hand, Tanzer's group has implicated CRT in regulating cell adhesion from the extracellular cell surface, as a putative mannoside lectin (White et al, 1995). CRT was reported to be a cell surface receptor that binds the mannoside peptides of the ECM protein, laminin, which in turn must bind to its cell surface receptors for cell spreading to occur. These authors show by immunofluorescence microscopy that CRT localizes to the cell surface of B16 mouse melanoma cells, and that the presence of anti-CRT antibodies in the medium prevents cell spreading.
Materials

Geneticin (G-418 sulfate), tissue culture media, trypsin, trypsin/EDTA and restriction endonuclease were from Gibco (Canadian Life Technologies, Burlington, ON, Canada). Fetal bovine serum (FBS) was from ICN Biomedicals (Costa Mesa, CA, USA). All the electrophoresis reagents were purchased from Bio-Rad (Richmond, CA, USA). Chemiluminescence ECL Western Blotting system was from Amersham (Oakville, ON, Canada). A mouse monoclonal antibody against vinculin was from ICN ImmunoBiologicals (Montreal, PQ, Canada), a mouse monoclonal antibody against α-catenin was from Transduction Laboratories (Lexington, KY, USA), a rabbit polyclonal anti-fibronectin antibody was from Collaborative Biomedical Products (Bedford, MA, USA), a rabbit polyclonal antibody against pp 125 focal adhesion kinase was from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA), mouse monoclonal antibodies against actin, β-catenin and cadherin were from Sigma (St. Louis, MO, USA). Anti-talin was from Dr. K. Burridge (University of North Carolina), anti-β1 integrin was from Dr. M. Ginsberg (Scripps Research Institute) and anti-α3 and anti-β1 integrins were from Dr. B. Chan (University of Western Ontario). All secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Fluorescein phalloidin was from Molecular Probes (Eugene, OR, USA). Liquid paraffin was from BDH (Poole, UK). Poly Mount was from Polysciences Inc. (Warrington, PA, USA). Vinol 205S
highest grade commercially available.

Cell Culture

Mouse L fibroblast stable transfectants (a gift from Dr. Marek Michalak, University of Alberta), KAB cells, CRT expressing about 2.0 fold elevation in CRT than control (referred to as CRT expressers), and BAK cells expressing about 0.5 fold reduction in CRT (referred to as CRT underexpressers) as determined by Western blot analyses (Burns et al., 1994a; Mery et al., 1996) were used in this study. A mock-transfected L fibroblast cell line, PGK (referred to as control) was used. All cells lines were grown in HG DMEM supplemented with 10% FBS and with Geneticin at a concentration of 100 µg/ml (50 µg/ml for PGK cells). A10 smooth muscle cell line, and stable transfectants, ACGF, expressing green fluorescent protein (GFP) tagged full length CRT, as described by Opas et al, 1996, as well as, stable transfectants of A10 cells and NIH/3T3 cells with truncated, minus leader sequence CRT (denoted cytosolic CRT) were grown in HG DMEM with 15% FBS, and transfectants with Geneticin at a concentration of 200 µg/ml after selection by FACS.

Cell Motility and Monolayer Migration

Cells were plated in HG DMEM and grown at a density of approximately 250,000 cells
per 50 mm φ cell culture dish for cell motility experiments. Cells were prepared for filming by changing medium to HG DMEM supplemented with 10% FBS plus 10mM Hepes buffer, which was then covered by a layer of liquid paraffin to prevent evaporation. Time-lapse recordings were done in a chamber kept at 37°C with a Hitachi CCD camera connected to an inverted phase contrast Olympus CK microscope. Images were collected at two minute intervals over a sixteen hour period using a frame grabber and digital image processor (Image-1, Universal Imaging, West Chester, PA, USA) on a Compaq 386/25 computer. Average velocities of cells were calculated by tagging and tracking cells (i.e., nuclear positions) for measurements of distances travelled over unit time. For each cell line 60 cells were followed over three two-hour intervals taken from three different filming sessions, after which ANOVA statistical analysis of the data was performed.

Cell monolayer migration studies were conducted as for cell motility, but cells were grown in a culture dish in the presence of a square coverslip (made by cutting approximately one third in length of a glass microscope slide (in order to add weight to the coverslip), until the dish was well packed. The coverslip was then removed and the monolayer edge was cleaned with a plastic scrubber before cells were prepared for filming. Images were collected at 15 minute intervals, then transferred to video tape.
Cells on coverslips were fixed in 3.8% formaldehyde in PBS for 10 min. After washing (3 x 5 min) in PBS, the cells were permeabilized with 0.1% Triton X-100 in buffer containing 100 mM PIPES, 1 mM EDTA and 4% (w/v) polyethylene glycol 8000 (pH 6.9) for 2 min, washed (3 x 5 min) in PBS and then incubated with either goat polyclonal anti-CRT antibody (diluted 1:50 in PBS) (Fliegel et al., 1989; Opas et al., 1991) or with a mouse monoclonal anti-vinculin antibody (diluted 1:50 in PBS) for 30 min at room temperature. After washing (3 x 5 min) in PBS, the cells were stained with appropriate secondary antibodies for 30 min at room temperature. The secondary antibodies were: fluorescein isothiocyanate-conjugated (FITC) donkey anti-mouse IgG(H+L) (diluted 1:30 in PBS), Texas Red-conjugated donkey anti-mouse (F(\text{ab}')\text{2} used at 1:30 dilution), and dichloro-triazinylamino-fluorescein-conjugated donkey anti-goat IgG(H+L) (diluted 1:30 in PBS). For double labelling, incubations with appropriate antibodies were done simultaneously. After the final wash (3 x 5 min) the slides were mounted in Vinol 205S which contained 0.25% 1,4-diazabicyclo-(2,2,2)-octane and 0.002% p-phenylenediamine to prevent photobleaching. For actin staining, a stock solution of 3.3 \mu M fluorescein phalloidin in methanol was diluted 1:10 in PBS and incubated with fixed and permeabilized cells for 20 min at room temperature. GFP-CRT fluorescence was recorded from either A10 cells or NIH/3T3 cells fixed with 3.8% paraformaldehyde in PBS. A Bio-Rad MRC-600 confocal fluorescence microscope equipped with a
microscopy.

SDS-PAGE and Western Blotting

Cells were homogenised in lysis buffer (50 mM Tris*Cl, 120 mM NaCl, 0.5% NP-40, pH 8.0) and frozen at -70°C. The amount of proteins in these extracts was determined by the method of Bradford (Bradford, 1976). Protein samples (10 µg/lane for extracts and 2 µg/lane for molecular weight markers) were subjected to SDS-PAGE as described by Laemmli (Laemmli, 1970). Subsequent transfer of proteins to nitrocellulose sheets and blocking of nonspecific sites with skim milk powder was carried out as described by Towbin (Towbin et al., 1979). Nitrocellulose sheets with bound proteins were incubated with primary antibodies for 1 hr at room temperature followed by incubation with horseradish peroxidase-conjugated donkey anti-mouse IgG(H+L) diluted 1:10000 for 1 hr at room temperature. The primary antibodies were used at the following dilutions in PBS: actin - 1:100, pp125 focal adhesion kinase - 1:200, cadherin, α-catenin, integrins and talin - 1:1000, vinculin - 1:1500, β-catenin, and fibronectin- 1:2000. Immunoreactive bands were detected with a chemiluminescence ECL Western Blotting system. The protein bands in each blot were scanned two dimensionally using a densitometer (UltroScanXL, Pharmacia) and areas under the curves were calculated using Gel Scan XL software.
Integrin Clustering

Integrin clustering was carried out as described by Leung-Hagesteijn et al, 1994 with and without modifications. A10 cells were plated sparsely on glass coverslips for a minimum of 24 hours, and were grown until coverslips were 50-75% confluent. Coverslips were washed with ice-cold HG DMEM (2 x 5 min), containing 10mM HEPES buffer, pH 7.2. The coverslips were incubated on ice with anti-α₃-integrin extracellular domain antibody (1:10) for 30 minutes. After the second washing (2 x 5 min) the live cells were incubated with FITC-conjugated donkey anti mouse IgG(H+L) at 37°C for 1Hr. Cells were then washed, fixed and immunolabelled with goat polyclonal anti-CRT antibody (diluted 1:50 in PBS), and secondary antibody dichloro-triazinylamino-fluorescein-conjugated donkey anti-goat IgG(H+L) (diluted 1:50 in PBS), as described in 'Immunostaining and Fluorescence Microscopy'. According to Leung-Hagesteijn, cell were permeabilized for 30 minutes with 0.1% triton-X-100 in PBS, and anti-goat antibody was diluted in 0.1% triton-x-100 in PBS.
Calreticulin is Localized to the ER and is Found in Neither Focal Adhesions Nor in α-Integrin Clusters.

CRT localization studies were carried out on A10 smooth muscle cells, as this cell type adheres, flattens out and forms strong well-defined focal adhesions. Therefore, both the ER and focal adhesion are distinctly visible and the localization of CRT can be well characterized. According to reports from the group of Shoukat Dedhar (Rojiani et al, 1991; Leung-Hagesteijn et al, 1994; Coppolino et al, 1995), CRT is hypothesized to bind to the α-integrin subunit, presumably within focal adhesions at a ratio of 1:1, as it does in vitro. Therefore, such substantial accumulation of the protein that should be detectable with the techniques used by us.

Indirect double immunofluorescence labelling of CRT and the adhesion protein, vinculin, demonstrate that CRT does not localize to the focal adhesions, but remains localized to the ER in A10 cells (Fig. 2). CRT specific staining (Fig. 2a) remains detectable only in the ER and fluoresces in a completely different pattern than vinculin (Fig. 2b). In most cases CRT distribution does not coincide with that of vinculin as shown in the merged image (Fig. 2c) although in some instances there is an overlay of signal due (discussed below).

To reproduce Leung-Hagesteijn et al (1994) results I conducted α-integrin clustering experiments with anti-α3-integrin antibody, since A10 cells express the α3β1
Fig. 2. CRT is not detectable in A10 cells in focal contacts by immunofluorescence after double labelling with antibodies against CRT (A) and vinculin (B). Confocal optical sectioning detects vinculin label in focal contacts at the substratum level (B). At the same optical plane CRT label, although detectable, follows entirely different pattern of distribution (A). C is a merged image A and B to show the cellular CRT distribution in comparison with that of vinculin. Scale bar = 25 μm.
Using an α-integrin clustering protocol described by Leung-Hagesteijn et al (1994),
followed by immunostaining carried out according to our standard procedure, there was
no detectable localization of CRT within integrin clusters (Fig. 3a-c). Therefore, the
exact labelling method that includes 30 min permeabilization in buffered Triton,
described by Leung-Hagesteijn et al, (1994), was followed. However, even in this case
I was unable to find CRT labelling specifically localized with integrin clusters (Fig. 3d-f).

In further attempts to demonstrate whether or not CRT is present in focal
adhesions, I utilized three cell lines expressing green fluorescent protein (GFP)-linked
CRT. These cell lines are described as follows: 1) A10 cells transfected with full length
ER-resident CRT linked with GFP; 2) A10 cells transfected with a truncated (minus
leader sequence) cytosolically-targeted CRT linked with GFP; and 3) NIH 3T3 cells
transfected with a truncated (minus leader sequence) cytosolic CRT linked with GFP.
Detection and localization of GFP-CRT was carried out by two techniques. Firstly,
localization was studied by double immunostaining of total cell CRT and vinculin (Fig.
4). In full length ER GFP-CRT-transfected cells did not co-localize CRT with vinculin
staining (Fig. 4a-c). Cells expressing truncated cytoplasmic GFP-CRT have CRT
labelling which can be seen to overlap with that of vinculin in some cases (Fig 4d-l).
However, as CRT has been targeted to the cytoplasm I would expect it to be detected
there. In order for CRT to be specifically localized in focal adhesions there must be
Fig. 3. CRT does not co-localize with α₅ integrin clustered by a specific antibody. A-C shows integrin clusters on the cell surface (A), CRT in the same cells after permeabilization for 2 minutes according to the standard procedure (B), and a merge of A and B in C. D-F shows integrin clusters on the cell surface (D), CRT in the same cells after 30 minutes of permeabilization as described by Leung-Hagesteijn et al, (1994) (E), and a merge D and E in F. Scale bar = 25 μm.
observed. Although, with merged images (Fig. 4 c, f, i) I find an overlay of signal in some cases, no specific detection of CRT in focal adhesions can be ascertained as discussed below.

The techniques of immunofluorescence staining and confocal microscopy limits the degree to which we can establish CRT is not present in focal adhesions. In all cases of immunofluorescence staining there is background staining due to secondary antibody non-specific labelling, above which CRT specific labelling must be detected. Also, with axial resolution limitations of the confocal microscope (~1μm) it is not possible to separate ER CRT signal from optical sections centred at the plane of the substratum, where maximal vinculin signal is detected. Therefore, there are situations where focal adhesions near the cell center are found under the ER and then seen in merged images as co-localization of vinculin and CRT.

The second technique involves utilizing interference reflection microscopy (IRM), which is a form of incident light interferometry in which radiance of the image is indicative of the distance separating the cells substratum from the cell undersurface (Opas et al, 1996a). As the cell’s undersurface comes closer to the substratum the blacker the image becomes, while the further the cells undersurface the lighter the image. For example, focal adhesions are seen as black streaks, while close contacts are seen as greyish areas (Burridge et al, 1988). Since GFP fluoresces, visualization of GFP-CRT with focal adhesions seen by IRM can be conducted on non-permeabilized cells, thus
Fig. 4. Cytosolically targeted CRT does not co-localize with vinculin in focal adhesions. A-C shows staining of GFP-tagged full length ER CRT with anti-CRT antibodies (A), anti vinculin staining (B) and merged image of A and B (C). ER CRT does not co-localize with vinculin. Cytosolically targeted GFP-tagged CRT is present in the cytoplasm (D, G) as detected by GFP fluorescence. Endogenous ER-resident CRT is detected in the same cells by antibody staining (D, G). Vinculin distribution in the corresponding cells is shown in E and H. F and I show merged images. D-F show A10 cells while G-I show NIH/3T3 cells. Scale bar = 25 μm.
there is no co-localization between CRT and focal adhesions (Fig 5a-c). As for cytosolic GFP-CRT, there is once again random cytoplasmic GFP fluorescence, however no distinct co-localization of GFP-CRT is seen within the focal adhesions (Fig. 5d-l).

CRT Expression Level Affects Cell Morphology, Cytoskeleton, and Motility.

Microscopical observation of CRT immunolabelling in overexpressers, underexpressers, and control cell lines (Fig. 6) also reveals changes in cell shape. With overexpressers of CRT (Fig. 6a) the ER pattern is clearly visible as the cells are more spread and seem larger in comparison to CRT underexpressers (Fig. 6b), where the CRT ER immunofluorescence labelling is not as defined since the cells are more rounded. Cell shape may reflect the adhesiveness of these cells, since the degree of spreading grown in culture is determined by degree of adhesion of cells to the substratum, to each other and the organization of the cytoskeleton.

Differences in adhesion can also be reflected in actin stress fiber formation. Actin stress fibres are a large structural component of focal adhesions and there is an increase in prominence of stress fibres in more stationary, adhesive cells (Jockusch et al, 1995). Fluorescein phalloidin labelling of actin in cells expressing different levels of CRT reveals an increase in stress fibre formation, as well as the appearance of more
Fig. 5. Cytosolically targeted CRT does not co-localize with focal contacts as revealed by IRM. A-C shows fluorescence of GFP-tagged full length ER CRT (A), focal contact distribution revealed with IRM (B) and merged image of A and B (C). ER CRT does not localize to focal contacts. Cytosolically targeted GFP-tagged CRT is present in the cytoplasm (D, G) as detected by GFP fluorescence. IRM images of focal contact distribution in the corresponding cells is shown in E and H. F and I show merged images. D-F show A10 cells while G-I show NIH/3T3 cells. Scale bar = 25 μm.
Fig. 6. Distribution of CRT in L fibroblasts differentially expressing CRT (A - overexpressers, B - underexpressers, and C - control) is very similar and the discernible differences can be attributed to the different degree of cell spreading. Scale bar = 25 µM.
more rounded with shorter and fewer stress fibres and fewer protrusions (Fig. 7b). Previous analysis of L fibroblast by (Opas et al, 1996a), found overexpressers to be quantitatively more spread, and due to stress fiber formation their cell shape deviates the most from that of a circle; the inverse is true for underexpressers. Therefore, an increase in CRT abundance in L fibroblasts corresponds to changes in cell adhesion-related processes such as cell shape and spreading.

Cell motility was also used as an assay reflecting cell adhesiveness, since it can be assumed that the degree of cellular adhesion will be inversely proportional to cellular velocity, the average speed of cell locomotion. This notion has been reinforced by the fact that focal adhesions are most prominent in stationary cells and can even be absent in highly migratory cells (Burridge et al, 1988). I found that the level of CRT expression is inversely proportional to cell motility (Fig. 8). Overexpressers of CRT move at the slowest velocity, $0.498 \pm 0.114 \mu m$ per min, while underexpressers were very motile and moved at the impressive velocity of $0.974 \pm 0.116 \mu m$ per min, almost twice as fast as the overexpressers. The transfection host cell line, L fibroblast cells and mock transfectants, PGK cells moved at velocities of $0.751 \pm 0.125 \mu m$ per min and $0.77 \pm 0.180 \mu m$ per min, respectively.
Fig. 7. Overexpression of CRT is associated with the appearance of prominent stress fibres and numerous filopodia as revealed by staining with a fluorescent F-actin probe, phalloidin. A - overexpressers, B - underexpressers, and C - control. Scale bar = 25 μm.
Fig. 8. Differential CRT expression affects velocity of locomotion of L fibroblasts. Each box represents one filming experiment in which cells were time-lapsed for 16 Hrs and 60 cells were tracked for 3 times 2 Hrs intervals. Each box represents the cell type by the coinciding box colour and name. All data for the groups of overexpressers and underexpressers are statistically different as calculated by ANOVA statistics, while they are not different within the cell type. All data for the groups of control or mock transfectants are not statistically different than underexpressers or overexpressers, although there is a distinguishable tendency of decreasing cellular velocity with increasing CRT expression.
Fibronectin Matrix Assembly is Proportional to the Level of CRT.

Fibronectin is an ECM protein that is recognized via its RGD amino acid sequence by integrins (Burdidge et al., 1988). The fibronectin receptor is the $\alpha_2\beta_1$ integrin heterodimer, and it is mainly the extracellular domain of $\alpha_2$-integrin subunit which is responsible for the ligand binding (Wu et al., 1993). L fibroblast cells express $\alpha_2\beta_1$ integrin (see below). De novo fibronectin assembly occurs at focal adhesion sites in a manner that is regulated by the level of cell surface $\alpha_2\beta_1$-integrins (Wu et al., 1993). Recent evidence reveals that binding of fibronectin to integrins is not sufficient for fibronectin fibril assembly, but intact focal adhesion in association with stress fibers must be present as well (Christopher et al., 1997). Therefore, de novo deposition of fibronectin matrix meshwork can be indicative of the degree of focal adhesion formation, which in turn is indicative of cell adhesiveness.

Figure 9, shows a dramatic difference in the deposition of fibronectin as overexpressers of CRT (Fig. 9a) assemble a more confluent and dense fibronectin matrix meshwork in comparison to both underexpressers (Fig. 9b) and the control cell line (Fig. 9c).

Vinculin-Rich Adhesions are More Abundant in L Fibroblast CRT Overexpressers Than in CRT Underexpressers.

Vinculin is a cytoskeletal adhesion protein, which is localized to both cell-ECM
Fig. 9. Differential CRT expression affects the degree of fibronectin matrix deposition. The most dramatic difference in the amount and density of the fibronectin matrix is seen between CRT overexpressers and the remaining cell lines. A - overexpressers, B - underexpressers, and C - control. Scale bar = 25 μm.
Control  

CRT Underexpressers  

CRT Overexpressers
adherens) (Otto, 1990). CRT overexpressers immunolabelled for vinculin show staining in both focal adhesions and in cell-cell junctions in densely populated long term cultures (Fig. 10a). With underexpressers of CRT, only few focal adhesions were seen with anti-vinculin staining (Fig. 10b). In comparison to underexpressers, overexpressers of CRT appear to have an epithelialized monolayer morphology.

Changes in CRT Abundance Correspond to Changes in the Abundance of Cytoskeletal Proteins, Vinculin and β-Catenin, and the Abundance of ECM Protein, Fibronectin.

Western blotting was used to measure the abundance of proteins. As seen in Figure 11a, vinculin protein expression is increased ~3 fold over the control line expression level, while the underexpressers showed a 50% reduction in signal in comparison to the control cell line. These results are in accordance with similar changes in vinculin mRNA abundance (Opas et al, 1996a).

Analysis of fibronectin protein levels by Western Blotting reveals an increased abundance of fibronectin in their protein lysates of CRT overexpressers (Fig. 11b). A similar correlation is observed with the control cell line and with CRT underexpressers, as there is less of fibronectin protein seen with the control cells than the overexpressers and an even lower abundance with underexpressers (Fig. 11b).
Fig. 10. CRT overexpression induces vinculin-rich cell-cell junctions in L fibroblasts. This figure shows immunolocalization of vinculin in crowded cultures of CRT overexpressers (A), underexpressers (B), and control cells (C). CRT overexpressers are flatter in comparison to the CRT underexpressing cells and develop abundant vinculin-rich cell-cell junctions thus assuming an epithelial cell sheet morphology. Scale bar = 25 μm.
Fig. 11. Effect of the differential expression of CRT on expression of vinculin (A) and fibronectin (B). The densitometric data are shown as percentage of values obtained for the control cells. The inserts show blots in which positions of lanes correspond to the graph bars. Standard deviation errors are as follows: vinculin: overexpressers (300 ± 127.8), underexpressers (48 ± 13.8), control (100 ± 12.4); and fibronectin: overexpressers (263 ± 93.4), underexpressers (39 ± 37.0), control (100 ± 24).
In an attempt to determine if the expression of other adhesion proteins is also co-regulated with CRT expression, we analysed the expression of $\alpha_5$-integrin and $\beta_1$-integrin amongst the different cell types (Fig. 12). No significant change in expression was observed. Other focal adhesion proteins analysed were actin, talin and pp125 focal adhesion kinase, all of which showed no change in protein abundance (Fig. 13).

As we observe an epithelial-like behaviour with our CRT overexpressers, zonula adherens specific proteins, $\alpha$-catenin and $\beta$-catenin were also examined (Fig. 14). The level of protein expression of $\beta$-catenin is increased ~2.6 fold CRT overexpressing fibroblasts, although the $\beta$-catenin level in CRT underexpressers is not significantly changed. No significant change is seen with $\alpha$-catenin protein levels.

**Overexpression of CRT Coincides With Enhanced Cell-Cell Interactions.**

A functional assay used to observe differences in cell-cell interactions with changing CRT expression is examination of monolayer migration. Cells were grown for time-lapse filming to a crowded state, at which point a coverslip was removed from the cell culture dish, the monolayer edge cleaned with a plastic scrubber and migration of cells recorded. Overexpressers and underexpressers demonstrated vastly different behaviour in cell migration (Fig 15). After 2hrs of filming cell migration the leading edge is visible and comparable in all cell types. As migration continues to 17hrs and
Fig. 12. Effect of the differential expression of CRT on expression of integrin subunits $\beta_1$ (A) and $\alpha_\delta$ (B). The densitometric data are shown as percentage of values obtained for the control cells. The inserts show blots in which positions of lanes correspond to the graph bars. Standard deviation errors are as follows: $\beta_1$-integrin: overexpressers (110 ± 27.3), underexpressers (93 ± 22.5), control (100 ± 16.0); and $\alpha_\delta$-integrin: overexpressers (118 ± 33.3), underexpressers (100 ± 36.4), control (100 ± 2.1).
Fig. 13. Effect of the differential expression of CRT on expression of actin (A), talin (B) and pp125FAK (C). The densitometric data are shown as percentage of values obtained for the control cells. The inserts show blots in which positions of lanes correspond to the graph bars. Standard deviation errors are as follows: actin: overexpressers (89 ± 7.0), underexpressers (75 ± 5.1), control (100 ± 7.7); talin: overexpressers (120 ± 9.6), underexpressers (128 ± 3.0), control (100 ± 2.7); and pp125FAK: overexpressers (112 ± 26.9), underexpressers (100 ± 4.7), control (100 ± 2.3).
Fig. 14. Effect of the differential expression of CRT on expression of $\alpha$-catenin (A) and $\beta$-catenin (B). The densitometric data are shown as percentage of values obtained for the control cells. The inserts show blots in which positions of lanes correspond to the graph bars. Standard deviation errors are as follows: $\alpha$-catenin: overexpressers ($87 \pm 10.8$), underexpressers ($73 \pm 14.8$), control ($100 \pm 5.6$); and $\beta$-catenin: overexpressers ($211 \pm 51.3$), underexpressers ($123 \pm 25.6$), control ($100 \pm 16.8$).
in underexpressers of CRT. The time-lapse video microscopy demonstrated that CRT overexpressers migrated in a sheet manner. In comparison, underexpressers migrated at a faster velocity, and they appear to migrate completely independently. The monolayer migration of the control cell line was comparable to that of the overexpressers.
Fig. 15. Differential expression of CRT affects motility pattern of L fibroblasts in an
in vitro scrape wound assay. A-C shows that CRT overexpressing cells migrate as a
sheet. D-F shows that CRT underexpressing cells migrate maintaining fairly substantial
cell-to-cell separation. G-I shows migration of control cells to be similar to CRT
overexpressers. While columns labeled 2 and 17 Hrs show the same fields of view, in
column labeled 27 Hrs the area has been moved forward to show advancing cells.
In summary, my data indicates that CRT does in fact play a role in modulation of cell adhesiveness, as an increase or a decreases in the abundance of CRT expression correlates with a coinciding increase or decrease in cellular adhesion in L fibroblast cell. Additionally, I find evidence contrary to Leung-Hagesteijn et al. (1994), who hypothesized CRT to function in this capacity as a cytoplasmic protein directly at the site of focal adhesions. L fibroblasts that either overexpress CRT or underexpress CRT or have been mock transfected were used as cell lines to examine the effects of differential CRT expression on cell adhesiveness. These cell lines are a good model to study the effects of changes in CRT expression because the differences in CRT expression levels in terms of protein abundance are still within the range reported for a variety of tissues (Khanna and Waisman, 1986). As previously reported, overexpressers of CRT exhibit increased efficacy of cell attachment to the ECM protein, fibronectin (Opas et al, 1996a). They also show enhanced cell spreading and formation of cell protrusions, as measured by cell area and degree of cell shape deviation from a circle respectively (Opas et al, 1996a). Underexpression of CRT causes inverse effects in stable cell lines (Opas et al, 1996a) and in transiently downregulated cells (Leung-Hagesteijn et al, 1994). Thus the purpose of the present investigation was to test the hypothesis that increased and decreased expression of CRT respectively correlates with an increase and a decrease in cellular adhesiveness.
In this study I have demonstrated that with differential expression of CRT in stable L fibroblast cell lines coordinate changes in cell adhesiveness occur. These changes in cell adhesion manifest as increases in both cell-substratum and cell-cell interactions in CRT overexpressers, while the inverse is the case for CRT underexpressers. One of the major issues addressed in this study is the subcellular localization of CRT in relation to focal adhesions, and subsequently, the hypothetical mechanism by which CRT may be able to modulate cell adhesiveness.

One hypothetical mechanism whereby CRT is proposed to modulate cell adhesiveness requires CRT-integrin interaction and thus CRT must be present in focal adhesions (Leung-Hagesteijn et al, 1994). By analogy to other focal adhesion proteins we would expect CRT to be present within all focal adhesions in such a quantity that it would be detectable by immunofluorescence microscopy. However, I have failed in all attempts to successfully localize CRT within these adhesion sites. A10 smooth muscle cells are very rich in cell-substratum attachments, they exhibit a high degree of stress fiber formation and are essentially non-motile in comparison to L fibroblasts. Using A10 cells and double immunofluorescence labelling for CRT and vinculin, as a focal adhesion marker (Otto, 1990), I was unable to co-localize the two. Additionally, by artificially inducing α-integrin clusters on the cell surface I was unable to duplicate the reported in vivo co-localization of integrins and CRT (Leung-Hagesteijn et al, 1994). As the CRT protein must be present in the cytosol to be able to interact directly with integrins, the presence of cytosolically targeted GFP-tagged CRT was examined in both A10 and
NIH3T3 cells. There was no discernible CRT localization to focal adhesions, as detected by GFP fluorescence or with anti-CRT immunofluorescence labelling, only a random cytosolic CRT signal was recorded.

In this study I have investigated several adhesion-dependent phenomena namely cytoskeletal organization, cell motility and ECM deposition. Overexpressers of CRT develop more prominent stress fibers than the control cells, while underexpressers in turn developed less as detected by fluorescent labelling for actin. Cell motility (understood here as average speed of cell locomotion) was used as an inverse indicator of cell adhesiveness. Overexpression of CRT correlated with a decrease in cell motility, while an increase in motility was associated with underexpression of CRT. My results are in accordance with previous reports of corresponding changes in cell motility in overexpressers and underexpressers of the adhesion plaque protein, α-actinin, where a decrease in motility is seen in overexpressers and an increase in underexpressers (Jockusch et al, 1995). Also, underexpression of vinculin was shown to increase cell motility and impair cell attachment (Jockusch et al, 1995). Fibronectin fibril meshwork formation is also indicative of cellular adhesiveness as the deposition of fibronectin fibrils requires the presence of fully formed (i.e., stress fiber-associated) focal contacts (Christopher et al, 1997). In cultures of the CRT overexpressers we see an increase in the deposition of fibronectin matrix and a corresponding decrease is detected in underexpressers. The fibronectin matrix deposition was also investigated by Western blot analysis, which revealed a considerable increase of fibronectin protein in the lysates.
of CRT overexpressers. As of yet it has not been determined what percentage of the fibronectin in the matrix is due to newly synthesized protein or to the deposition of fibronectin present in the cell culture medium (HG DMEM plus 10% FBS). Considering the vast abundance of fibronectin in FBS it is likely that the culture medium provides most of the fibronectin which forms the matrix meshwork.

Increases in cell-cell interactions in addition to increases in cell-substratum interactions were observed in overexpressers of CRT as they exhibit epithelial-like behaviour in cell culture. The CRT overexpressers, although being transformed fibroblasts, are able to form monolayers which, during cell culture manipulations, detach in a sheet-like manner and maintain sheet integrity after detachment, i.e., behave like epithelial cell sheets. Immunofluorescence labelling of the cytoskeletal protein, vinculin, which is present in both cell-substratum and cell-cell adherens-type junctions is drastically increased at these junction sites in the CRT overexpressers compared to either CRT underexpressers or control cells. Western blot analysis of cell extracts shows that there is a substantial increase in vinculin abundance in overexpressers of CRT while lower abundance of vinculin is recorded in CRT underexpressers. Interestingly, in terms of focal adhesions, the effect of CRT overexpression appears to be "vinculin-specific" as the level of other focal adhesion proteins thus far examined: \( \alpha_2 \)-integrin, \( \beta_1 \)-integrin, actin, talin, and pp125FAK remain unchanged. Therefore, my data comprise considerable evidence that changes in CRT expression correlate with coinciding changes in vinculin expression, and in turn, in cell adhesion. These protein data are further
Using a wound healing time-lapse cell motility assay where the leading edge of a cell monolayer was filmed, I obtained an additional evidence correlating changes in CRT expression with corresponding changes in cell adhesiveness as assessed by cell motility. I found that underexpressers of CRT display a rather dramatic "anti-social behaviour" as they completely dissociate from the wound edge and rapidly move in random directions. The CRT overexpressers retain cell-cell contacts and migrate in an organized uniform manner maintaining a cell sheet appearance over a considerable distance away from the wound edge, which I refer to as the epithelial-like-behaviour. Western blot analysis of adherens junction proteins revealed that in overexpressers of CRT there is an increase in the abundance of β-catenin, while the abundance of actin and α-catenin was not significantly changed. In summary, these data show that an increase or a decrease in the expression of CRT is coincidental with an increase or a decrease in the expression of several adhesion-related proteins which is most pronounced in the L cell line overexpressing CRT. The increase in the abundance of these proteins is most likely responsible for the increased adhesiveness of cells overexpressing CRT, while the inverse is true for vinculin abundance in the underexpressers of CRT. Therefore, I hypothesize that overexpression or underexpression of CRT induces corresponding increases or decreases in the expression of vinculin and β-catenin, and thereby modulates adhesiveness.
This conclusion is supported by recent experiments performed in Dr. Opas' laboratory that indicate that, functionally, the level of CRT expression correlates with an ability of cells to aggregate at a given shear stress. Aggregates of CRT overexpressers are highly cohesive, aggregates of control cells have average cohesiveness, while there is low cohesiveness in CRT underexpressers. The aggregates formed by CRT overexpressers stained weakly but specifically for anti-N-cadherin at cell-cell junctions while specific staining could not be detected in aggregates of other cell lines (Opas, unpublished results). Further more the increase in β-catenin abundance in CRT overexpressers coincides with a 10 fold increase in N-cadherin mRNA expression as well as an increase in N-cadherin protein expression (personal communication of Dr. Orest Blaschuk).

**HOW IS CRT ABLE TO MODULATE CELL ADHESIVENESS?**

As outlined in the Introduction, while it has been reported that CRT performs many functions, only a few of these are unequivocally demonstrated. And thus, (I) CRT stores Ca\(^2+\) and is (II) a chaperone, it (III) affects glucocorticoid-sensitive gene expression and (IV) cell adhesion. Also, (V) CRT localizes to the ER, where all the other proposed functions and localizations of CRT are so far hypothetical. Because CRT is a multifunctional protein, its numerous functions must be considered in assessing in what capacity CRT is able to regulate cellular adhesion.
Is CRT, in its Capacity as a Calcium Storage/Buffering Protein Able to Affect Cytosolic Ca\(^{2+}\) Concentration and, Indirectly, Cell Adhesiveness?

Cellular adhesion has been shown to be sensitive to cytosolic Ca\(^{2+}\) concentrations (Sjaastad and Nelson, 1997). Due to this fact, the Ca\(^{2+}\)-storage/buffering function of CRT should be considered to be involved in the regulation of cellular adhesion. However, it has been shown that, in standard tissue culture conditions, cytosolic Ca\(^{2+}\) levels remain the same irrespective of the level of CRT expression (Bastianutto et al, 1995; Mery et al, 1996; Opas et al, 1996a), including CRT’s ablation by homologous recombination (Coppolino et al, 1997). Stimulation of L fibroblast cells expressing differential levels of CRT with bFGF induces cytosolic Ca\(^{2+}\) transients which are similar in magnitude (Opas et al, 1996a). This is evidence that CRT does not affect calcium signalling in these cells. It has been postulated that only under adverse conditions, such as growing cells in at very low Ca\(^{2+}\) concentration, or conversely, flooding the cytoplasm with Ca\(^{2+}\) by exposing cells to media containing high Ca\(^{2+}\) concentration in the presence of a Ca\(^{2+}\) ionophore, ionomycin, that the level of expression of CRT plays a role by rescuing the CRT overexpressers from growth inhibition or death (Opas et al, 1996a).

Although an increase in CRT results in an increase in ER Ca\(^{2+}\) storage capacity, it has been argued that the free Ca\(^{2+}\) levels in the ER are the same in both overexpressers and control cell lines (Mery et al, 1996). Even if the free Ca\(^{2+}\) levels within the ER were
different, this does not appear to translate into different concentrations of Ca\(^{2+}\) in the cytosol of cells differentially expressing CRT. Therefore, the available evidence does not support the idea that CRT can regulate adhesion via an effect on cytoplasmic Ca\(^{2+}\) concentration.

**Is CRT, as an ER Chaperone Protein Able to Modulate Cell Adhesiveness?**

CRT as a chaperone has not been shown to interact with any adhesion-related proteins. Both vinculin and β-catenin are cytosolic proteins, thus are not processed through the ER. Also, CRT has only been shown to interact with α-integrin via its GFFKR amino acid sequence (Rojiani et al, 1991), but this sequence is not oriented in the ER lumen during α-integrin protein processing. To date there has been no observed association of CRT with N-cadherin as a chaperone or otherwise, thus any possible interaction between these proteins remains to be investigated. If CRT chaperone activity is able to translate into coordinate changes in cellular adhesion by its interaction, as a chaperone, with N-cadherin, then it still remains to be explained how this translates into induction of N-cadherin mRNA transcription. Therefore, I believe that CRT in its capacity as a molecular chaperone is not significantly contributing to regulation of cell adhesion.
Does CRT Mediate Cellular Adhesion as a Cell Surface Lectin Receptor?

In order for CRT to function as a surface lectin it must be secreted. Because CRT is not a transmembrane protein it would require also a mechanism to be maintained at the cell surface. As detailed in CRT retention/escape studies, CRT is mostly found to be retained in the ER and the minimal amount of extracellular CRT is thought to be present as a result of cell death naturally occurring in cell culture (Sönnichsen et al, 1994). Also, the reported photomicrographs purportedly localizing CRT to the cell surface (Gray et al, 1995; White et al, 1995) are of such low quality that it precludes drawing any conclusions as to its localization there. Consequently, the “CRT-as-a-cell-surface-lectin” has been considered as a truly unlikely option for the protein to regulate cell adhesiveness. However, to satisfy my curiosity, one-time experiments were conducted to look into this possible mode of CRT activity. Cell surface anti-CRT immunofluorescence staining was performed on L fibroblasts differentially expressing CRT, as well as attempts to prevent adhesion with the presence of extracellular anti-CRT antibodies. Next, the relative amount of CRT protein present in the cell culture medium after incubation with cells growing in culture for several days was compared between cell types expressing different levels of CRT. Finally, cells were maintained in the presence of extracellular CRT added at 100 µg/ml into the growth medium. In all cases there was no evidence to support the hypothesis that CRT modulates cellular adhesion.
Furthermore, if adhesion-related effects were caused by overexpression of CRT compromising its ER retention mechanisms and the protein finding its way to the extracellular side of the cell surface where it played an adhesive role, this could not explain the decrease in cell adhesiveness which accompanies CRT underexpression.

**Does CRT Modulate Cell Adhesion by Direct Binding to α-Integrin in Focal Adhesions or α-Integrin Clusters?**

As biochemical evidence first pointed to the *in vitro* interaction of CRT with α-integrins, it was hypothesized by Dedhar’s group that this interaction is also realized *in vivo* (Rojiani *et al*, 1991). For this to occur there must be cytoplasmic CRT. However, even at this time, the failure to detect CRT within focal adhesions sites was reported by these authors (Rojiani *et al*, 1991). To date immunolocalization studies failed to detect CRT in either the cytoplasm or in the focal contacts (Bastianutto *et al*, 1995; Michalak *et al*, 1996; Opas *et al*, 1996a). CRT has also not been detected in the cytosol by pulse-chase experiments in the studies of CRT retention in the ER (Peter *et al*, 1992; Sönnichsen *et al*, 1994). Additional *in vitro* biochemical evidence points to a 1:1 ratio of CRT binding to α-integrins (Coppolino *et al*, 1995). Thus the degree of cell adhesion was hypothesized to be proportional to the level of CRT-α-integrin interactions. With this in mind the only *in vivo* co-localization of CRT with α-integrins has been
demonstrated in human prostate carcinoma cells, by cell surface \( \alpha \)-integrins clustering experiments (Leung-Hagesteijn et al, 1994). This procedure brings a number of considerations to mind. Firstly, as this cell type is only weakly adhesive and it does not form focal adhesions, is \( \alpha \)-integrin clustering in these cells an appropriate method to examine mechanisms of cell adhesion? Secondly, considering the technical limits of resolution in fluorescence microscopy, it is intriguing to note that the ER is not visible in the micrographs of Leung-Hagesteijn et al. (1994) which show labelling with the same anti-CRT antibody that I used throughout this study. This points to possible methodological errors in this study. Lastly, as I do not detect co-localization between CRT and \( \alpha \)-integrins when I repeat the experiment of Leung-Hagesteijn et al. (1994) using anchorage dependent cell lines, it suggests that the positive results of these authors are perhaps unique to human prostate carcinoma cells.

Experiments in which double labelling with anti-CRT and anti-vinculin antibodies were performed clearly show that patterns of distribution of the two labels are different. The cases when I see an overlap of the labels are most likely due to the spatial overlap of antigens along the optical axis of a microscope and not true co-localization. Due to a limitation in axial resolution of a confocal microscope \((-1 \mu m)\) this cannot be decided conclusively. However, the vast majority of the overlap is seen under areas containing the ER; if it were a true co-localization one must then postulate a mechanism ensuring selectivity of CRT association with focal contacts in that the protein would “prefer” the centrally located focal contacts and “avoid” more peripherally located focal contacts.
present in the "leftover" ventral surfaces of cells that were torn off the glass during the sample preparation.

If CRT were present in focal contacts its detection there should not be difficult at all as CRT has been postulated to bind integrins with 1:1 stoichiometry (Coppolino et al, 1995). Using cells overexpressing the ER-resident full length GFP-CRT (i.e., with signal sequence and KDEL ER localization signal) allowed me to follow CRT in lining cells: it is not detected outside of the ER. Using cells overexpressing a cytoplasmically-targeted GFP-CRT (i.e., without signal sequence and KDEL ER localization signal) allowed me to follow CRT in the cytoplasm of living cells: it is not found in focal contacts visualized with either IRM or by vinculin labeling. Furthermore, targeting of purified CRT to the cytoplasm by microinjection has no effect on cell morphology, cytoskeleton or cell adhesion (Opas, unpublished data).

CRT was hypothesised to modulate cellular adhesion by specific, 1:1 binding to the GFFKR sequence of α-integrins (Dedhar, 1994). Therefore, I believe that CRT should be present in focal adhesions as seen with all other adhesion proteins that either make up or regulate focal adhesions. Since I did not find this to be true, I conclude that cytoplasmic, focal contact-associated CRT, if it exists, is both microscopically not-detectable and non-functional in terms of affecting cell adhesion. Thus I feel that I have circumstantial but solid evidence that CRT does not modulate cell adhesiveness by directly binding to α-integrins in focal adhesions.
Does CRT Modulate Cell Adhesiveness Via “Regulation” of Vinculin, N-cadherin and β-catenin From the Lumen of the ER?

All my morphological data indicate that CRT remains localized in the ER lumen and, if it affects adhesiveness, it is from the ER that the cell adhesion is modulated. Regulation of the ER form of CRT is associated with coordinate regulation of vinculin and N-cadherin and their respective RNAs. β-catenin, a cytoskeletal link protein for N-cadherin, is upregulated in cells stably overexpressing CRT. Neither actin, talin, pp125FAK, α5-integrin, β1-integrin nor α-catenin are regulated in a CRT-dependent manner. Regulation of the level of vinculin and β-catenin/N-cadherin is a sufficient requirement to account for all cell-substratum and cell-cell adhesion-related effects observed (Coll et al, 1995; Goldmann et al, 1995; Rodríguez Fernández et al, 1992; 1993; Volberg et al, 1995); but this, obviously, does not exclude involvement of other, yet unidentified proteins. In another series of experiments, transfection of L fibroblasts stably underexpressing CRT with a CRT expression vector containing cDNA encoding full length protein fully restored the level of both CRT and vinculin back to normal (Opas and Dziak, unpublished data). When retinal pigment epithelial cells stably underexpressing CRT were transfected with a full length CRT cDNA, again a coordinate increase both in the level of the ER-resident CRT and the level of vinculin in the transfectants was detected (Opas and Dziak, unpublished data). Finally, induction of overexpression of CRT in the Tet-on double-stable KN1 cell line was accompanied by
a ~2-fold increase in vinculin and ~2-fold increase in N-cadherin (Fadell and Opas, unpublished data). Thus, the level of expression of the ER-resident CRT affects the level of expression of a spectrum of proteins involved in cell adhesion. This scenario derives further indirect support from the fact that, in vivo, only the ER form of CRT inhibits steroid-mediated gene expression while its cytosolic variant is ineffective (Michalak et al., 1996). Furthermore, so far the examined effects are CRT-specific as overexpression of close relatives of CRT, calnexin and calsequestrin (Michalak et al., 1996), do not reproduce effects of CRT overexpression. Collectively, none of the effects of differential CRT expression require the protein to be present at the cytoplasmic face of the plasma membrane. Because the adhesion-related effects due to differential expression of the ER-resident CRT can be explained by its effects on vinculin/β-catenin/N-cadherin expression, it is likely that it is the ER-resident CRT which affects cellular adhesiveness. Therefore, it has been hypothesized that CRT in vivo may perform some of its functions from within the ER lumen (Krause and Michalak, 1997).

What Is The Nature Of The Switch That Activates CRT Signaling Within The ER?

Presently this can only be speculated on. For example, in vitro studies in Dr. Michalak’s laboratory demonstrated a direct Ca\(^{2+}\)-dependent interaction between CRT and PDI (Baksh et al, 1995a). CRT binds reversibly to PDI at low Ca\(^{2+}\) concentration
Binding of PDI abolishes Ca\(^{2+}\) binding to the high affinity site in P-domain of CRT and inhibits PDI's chaperone activity. As Ca\(^{2+}\) concentration increases the two proteins dissociate. Free CRT may associate with intraluminal CRT-binding proteins ("ER CRT receptors") which may be involved in signaling similar to that described for the GRP78 receptor (Mori et al, 1993; Cox et al, 1993). It may be via this hypothetical CRT receptor residing within the ER that ER-to-nucleus signaling may be initiated and, in turn, modulate cell adhesiveness by affecting expression levels of several adhesion-relevant proteins. Thus, the intraluminal CRT-PDI interaction can serve as an example of a possible, although entirely speculative, toggle switch for CRT signaling.

**How Can Cells Respond To Messages From The Lumen Of The ER?**

There are several pathways that may be used in CRT-dependent phenomena. These may include, for example, activation of transcription factors, specific protein phosphorylation by serine/threonine or tyrosine protein kinase or activation of a specific phosphatase. For example, cells respond to overexpression of misfolded proteins in the ER lumen by inducing expression of ER chaperone genes (McMillan et al, 1994). Consequently, a message must be transduced from the lumen of the ER to the nucleus to activate a specific response. This pathway has been studied extensively in yeast and shown to depend on an ER resident protein kinase activity (Shamu and Walter, 1996;
McMillan et al., 1994; Mori et al., 1993; Cox et al., 1993). However, there is no evidence for these kinases in mammalian cells (Pahl and Baeuerle, 1997a; Cao et al., 1995a). An ER-nucleus signaling pathway is involved in cellular cholesterol homeostasis via sterol regulated proteolysis of ER membrane-bound transcription factors called sterol-regulatory element binding proteins (Pahl and Baeuerle, 1997a; Wang et al., 1994). Another ER-nucleus pathway involves the transcription factor, NF-κB (Pahl and Baeuerle, 1997b; Pahl and Baeuerle, 1997b; Pahl and Baeuerle, 1995b). Using a DNA mobility shift assay Dr. Michałak found no evidence that changes in expression of CRT induce activation of NF-κB (personal communication). Therefore, CRT-dependent signaling occurs via pathways that do not involve activation of NF-κB. Considering reports of ER-specific, phosphorylation-dependent signaling (Shamu and Walter, 1996; Cox et al., 1993; Mori et al., 1993; Rindress et al., 1993; Prostko et al., 1995; Cao et al., 1995; Brostrom et al., 1995) it is highly likely that protein phosphorylation / dephosphorylation events are involved in CRT-dependent effects. Pilot immunoblotting experiments done in Dr. Opas’ laboratory detected a reduction in the phosphotyrosine (P-Tyr) signal and a change in profile of P-Tyr proteins in all cell lines overexpressing CRT (personal communication, Dr. Michal Opas). This, for example, might affect the rate of proteolytic cleavage of N-cadherin (to NCAD90) affecting the abundance of full length transmembrane form of N-cadherin (Lee et al., 1997; Lagunowich and Grunwald, 1991).
REFERENCES


Opas, M., E. Dziak, L. Fliegel, and M. Michalak. 1991. Regulation of expression and intracellular distribution of calreticulin, a major calcium binding protein of nonmuscle...


