The cell adhesion molecule C-CAM is a substrate for tissue transglutaminase

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Abstract C-CAM, a ubiquitously expressed cell adhesion molecule belonging to the carcinoembryonic antigen family, appears as two co-expressed isoforms, C-CAM-L and C-CAM-S, with different cytoplasmic domains, that can form homo-dimers in epithelial cells. In addition, C-CAM-L has been found in large molecular weight forms suggesting posttranslational, covalent modification. Here we have investigated the possibility that the cytoplasmic domain of C-CAM-L can act as a transglutaminase substrate. Glutathione *S*-transferase fusion proteins of the cytoplasmic domains of rat and mouse C-CAM-L as well as free cytoplasmic domains, released by thrombin cleavage from the fusion proteins, were converted into covalent dimers by tissue transglutaminase. These results demonstrate that the cytoplasmic domains of rat and mouse C-CAM-L are substrates for tissue transglutaminase, and lend support to the notion that higher molecular weight forms of C-CAM-L are formed by transglutaminase modification.

Key words: C-CAM; Biliary glycoprotein; Cell adhesion; Transglutaminase

1. Introduction

Rat C-CAM (rC-CAM), together with the homologous human and mouse biliary glycoproteins (hC-CAM and mC-CAM) belong to the carcinoembryonic antigen (CEA) subgroup of the immunoglobulin (Ig) superfamily [1]. Alternative splicing of these highly glycosylated, transmembrane proteins generates a number of isoforms which contain either 71–73 amino acid (C-CAM-L) or 10–12 amino acid long (C-CAM-S) cytoplasmic domains [1]. Both the long and short cytoplasmic domain isoforms have been shown to mediate intercellular adhesion [2]. The long cytoplasmic domain isoforms can be phosphorylated on tyrosine residues [3–5] and have been demonstrated to bind tyrosine kinases of the src family [3] and the tyrosine phosphatases SHP-1 and SHP-2 [1,5] supporting the notion that these proteins are important transducers or regulators of cellular signals. Both rC-CAM-L and mC-CAM-L, also known as Bgp1, have been shown to act as suppressors of tumour growth [6,7].

We have recently demonstrated that C-CAM is organised as non-covalently linked dimers within the membranes of epithelial cells [8]. In addition, the detection of C-CAM-L-containing species with apparent molecular masses twice that of monomeric C-CAM, in extracts of rat liver plasma membranes [8,9], led us to propose [8] that a population of C-CAM-L dimers may become covalently modified, perhaps by the action of tissue transglutaminase.

Transglutaminases (EC 2.3.2.13) are a group of enzymes which catalyse a Ca^{2+}-dependent acyl transfer reaction in which amide bonds are formed between γ-carboxamide groups of peptide-bound glutamine residues and primary amines [10]. This commonly results in the formation of proteolytically resistant, γ-glutamyl-ε-lysine isopeptide bonds within or between proteins. Transglutaminases have been implicated in diverse cellular processes, including covalent cross-linking of fibrin clots [11], formation of the cornified envelope of keratinocytes [12], stabilisation of extracellular matrices [13–15], formation of a detergent-insoluble protein scaffold in cells undergoing apoptosis [16], and regulation of cellular growth [17]. Although little is known about the endogenous substrates of tissue transglutaminase, which is found in all cells studied, it has been demonstrated that this enzyme can be activated by a physiological increase of the intracellular calcium concentration. Treatment of A431 cells with epidermal growth factor led to calcium-triggered transglutaminase cross-linking of the intracellular protein lipocortin I [18], and in hepatocytes tissue transglutaminase caused downregulation of the receptor for hepatocyte growth factor [17]. Both primary structure and conformation appear to determine whether proteins can act as transglutaminase substrates, with reactive glutamines often located on solvent-exposed or flexible areas of the protein [10,19]. Thus, while there is no consensus sequence for transglutaminase-catalysed modification, the two glutamine residues present in the cytoplasmic domain of rC-CAM-L have features often found in known transglutaminase substrates [15,19] in that they are located immediately adjacent to one another and close to a number of charged and polar amino acids.

Using recombinant proteins consisting of the cytoplasmic domain of C-CAM-L fused to glutathione *S*-transferase (GST), we now demonstrate that C-CAM-L is a substrate for tissue transglutaminase and that this reaction results in the formation of covalently linked homo-dimers of C-CAM-L.

2. Materials and methods

2.1. Reagents

Guinea pig liver transglutaminase, monodansylcadaverine (MDC), cystamine and thrombin were purchased from Sigma. A rabbit polyclonal anti-peptide antibody (L2), raised against a sequence present in the cytoplasmic domain of rC-CAM-L, has been described previously [8] and reacts with both rC-CAM-L and mC-CAM-L. A polyclonal antibody against GST was from Pharmacia and molecular mass standards for SDS-PAGE were from Bio-Rad.

2.2. Preparation of recombinant proteins

The construction of recombinant GST-mC-CAM-L (GST-Bgp1) using the pGEX-2T vector has been described previously [5]. GST-

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rC-CAM-L was made by inserting the cytoplasmic domain of rC-CAM-L from the pRAX vector [2] into a pGEX-2T vector (Pharmacia). For this purpose the forward primer 5′-AGAGGATC-CAATGGCTACTTCCTTTATTCCAGG-3′ and the backward primer 5′-AGCTGCGAATTCAATGTCACTTCTTTTT-3′ were used for amplification of the cytoplasmic region of rC-CAM-L by PCR using the pRAX vector as template. The PCR product was cut with *Bam*HI and *Eco*RI and ligated into the corresponding sites within the pGEX-2T vector. This insert encodes amino acids 445–519 of the rC-CAM-L. Proteins were produced in *Escherichia coli* BL21. Protein synthesis was induced by IPTG (0.2 mM). Purification of the GST fusion proteins was performed by affinity adsorption on glutathione-Sepharose according to a standard protocol from the manufacturer (Pharmacia).

2.3. Thrombin cleavage of fusion proteins

Fusion proteins in Tris-buffered saline (TBS), pH 7.4, were cleaved by incubation with thrombin (10 units/mg protein) for 4 h at room temperature, followed by dilution with TBS, pH 7.4, to the appropriate concentration for transglutaminase treatment.

2.4. Transglutaminase treatment of recombinant proteins

Samples (20 μl) of GST alone, GST-rC-CAM-L or GST-mC-CAM-L (1 mg/ml) in TBS, pH 7.4, containing either 5 mM $CaCl_2$ or 5 mM EDTA and varying amounts (5–20 mU) of guinea pig liver transglutaminase were incubated at 37°C for 30 min. In some experiments, the transglutaminase inhibitors MDC (0.5 mM) or cystamine (1 mM) were included in the reaction mixtures. Reactions were stopped by the addition of an equal volume of 2×SDS sample buffer [20] containing 10 mM DTT and samples were incubated at 100°C for 5 min prior to SDS-PAGE.

2.5. SDS-PAGE and immunoblotting

SDS-PAGE was performed according to Laemmli [20] and proteins were detected by staining with Coomassie brilliant blue or immunochemically, as described previously [8]. Immunoreactive proteins were visualised using an enhanced chemiluminescence (ECL) detection system (Amersham).

3. Results and discussion

To investigate the possibility that C-CAM-L could act as a substrate for tissue transglutaminase we prepared recombinant proteins consisting of the cytoplasmic domains of rat and mouse C-CAM-L fused to GST. After purification, GST appeared on Coomassie blue-stained gels as a 29 kDa band (Fig. 1, lane 1), while both GST-rC-CAM-L (Fig. 1, lane 2) and GST-mC-CAM-L (Fig. 1, lane 3) migrated as single bands, whose apparent M_rs of about 36 kDa were consistent with their expected values.

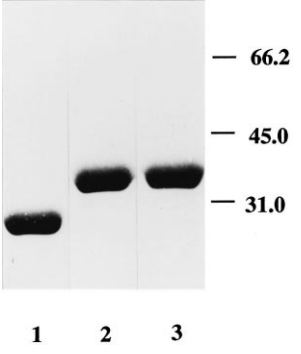

Fig. 1. SDS-PAGE analysis of recombinant proteins. Purified GST (lane 1), GST-rC-CAM-L (lane 2) and GST-mC-CAM-L (lane 3) were electrophoresed on 8% polyacrylamide gels and stained with Coomassie brilliant blue. The migration positions of M_r markers are indicated.

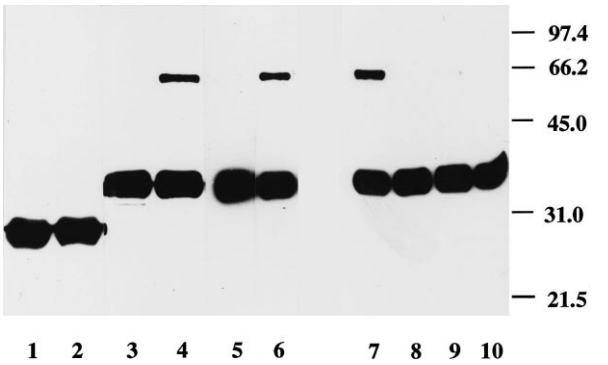

Fig. 2. Transglutaminase treatment of recombinant proteins. GST (lanes 1 and 2), GST-rC-CAM-L (lanes 3 and 4) and GST-mC-CAM-L (lanes 5 and 6), in TBS, pH 7.4, containing 5 mM $CaCl_2$ were incubated without (lanes 1, 3 and 5) or with (lanes 2, 4 and 6) 10 mU transglutaminase for 30 min at 37°C, subjected to SDS-PAGE and immunoblotted with anti-GST antibody (lanes 1 and 2) or antibody L2 (lanes 2–6). GST-mC-CAM-L in TBS, pH 7.4, containing either 5 mM $CaCl_2$ (lanes 7, 9 and 10) or 5 mM EDTA (lane 8) was incubated with transglutaminase alone (lanes 7 and 8) or together with the transglutaminase inhibitors MDC (lane 9) or cystamine (lane 10) and analysed with antibody L2. The migration positions of M_r markers are indicated.

It has been reported that GST appears as a dimer under native conditions [21], and after chemical cross-linking with 3,3′-dithiobis(sulphosuccinimidyl) propionate (DTSSP) we also observed that the majority of the GST molecules migrated at the position of dimers in SDS-PAGE (data not shown). However, GST migrated as a monomer both prior to (Fig. 2, lane 1) and after (Fig. 2, lane 2) treatment with transglutaminase. In contrast, addition of transglutaminase to GST-rC-CAM-L (Fig. 2, lane 3) resulted in the appearance of an additional band (Fig. 2, lane 4), whose apparent M_r of 70 kDa indicated that it was a dimeric form of the fusion protein. rC-CAM-L has only two glutamine residues in its cytoplasmic domain [1]. They are immediately adjacent to each other at positions 496 and 497, and could serve as acyl donors in the transglutaminase reaction. The conservation of these two glutamine residues in both human and mouse C-CAM-L [1] suggested that these proteins would also act as transglutaminase substrates. Indeed, addition of transglutaminase to a recombinant protein consisting of GST fused to the long cytoplasmic domain of mC-CAM-L (Fig. 2, lane 5) also resulted in the appearance of a 70 kDa band (Fig. 2, lane 6). These results suggest that the cytoplasmic domain of C-CAM-L acts as a substrate for tissue transglutaminase and indicate that the observed cross-linking is not the result of stabilisation of GST dimers.

Transglutaminases are Ca^{2+}-dependent enzymes [10], and cross-linking of both GST-rC-CAM-L (not shown) and GST-mC-CAM-L (Fig. 2, lane 7) was critically dependent upon the presence of Ca^{2+} and abrogated by the inclusion of EDTA in the reaction buffer (Fig. 2, lane 8). Cross-linking of the fusion proteins was also blocked by both MDC (Fig. 2, lane 9) and cystamine (Fig. 2, lane 10), two structurally unrelated transglutaminase inhibitors.

While transglutaminases show considerable specificity towards acyl donor glutamine residues in substrate proteins, they appear to be much less selective towards amine donor lysines [19]. Our results did not therefore allow us to predict if cross-linking occurred between cytoplasmic domains alone or

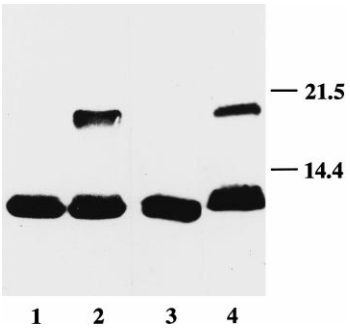

Fig. 3. Transglutaminase treatment of cleaved recombinant proteins. GST-rC-CAM-L (lanes 1 and 2) and GST-mC-CAM-L (lanes 3 and 4) in TBS, pH 7.4, containing 5 mM $CaCl_2$ were cleaved with thrombin and incubated without (lanes 1 and 3) or with (lanes 2 and 4) transglutaminase, prior to immunoblotting with antibody L2, as described in Fig. 2A. The migration positions of M_r markers are indicated.

involved the participation of the GST fusion partner. However, failure to alter the extent of transglutaminase-dependent cross-linking by addition of increasing amounts of free GST (not shown) suggested that amide bond formation likely occurred between cytoplasmic domain polypeptides.

The recombinant fusion proteins used in this study contain a thrombin cleavage site, allowing the release of the constituent GST and cytoplasmic domain moieties, which enabled us to assess directly the ability of the cytoplasmic domain to act as a transglutaminase substrate. Thrombin cleavage of GST-rC-CAM-L gave rise to a single cytoplasmic domain polypeptide with an apparent M_r of 7–8 kDa, recognised by antibody L2 (Fig. 3, lane 1). Upon addition of transglutaminase in the presence of Ca^{2+}, an additional band of about 16 kDa was detected (Fig. 3, lane 2) consistent with formation of dimers of the cytoplasmic domain. Transglutaminase treatment of thrombin-cleaved GST-mC-CAM-L (Fig. 3, compare lanes 3 and 4) gave identical results. Taken together, our data clearly demonstrate that the cytoplasmic domain of C-CAM-L acts as a substrate for tissue transglutaminase. Since the major products of this reaction are covalently cross-linked dimers of the cytoplasmic domain, these results further support our suggestion that mutual interactions between cytoplasmic domain sequences contribute to homo-dimerisation of C-CAM-L within plasma membranes [8]. However, the ability of the short cytoplasmic domain isoform, C-CAM-S, to form homo-dimers [8] suggests that the ectodomain is also involved in this reaction. This notion is supported by our unpublished observations that a soluble form of the ectodomain of rC-CAM can dimerise.

Tissue transglutaminase, which can be activated by e.g. growth factor-induced increase of the intracellular calcium concentration [18], is abundantly expressed in liver [10] and it is of particular interest that it is in this tissue that we [8] and others [9] have identified a number of high molecular weight C-CAM-L-containing species. The abundance of a group of proteins with an apparent M_r twice that of monomeric C-CAM, coupled with our demonstration that C-CAM is distributed as non-covalently linked dimers within plasma membranes, led us to propose that a population of C-CAM-L dimers [8] may become covalently modified. The demonstration, in the present study, that C-CAM-L acts as a substrate for transglutaminase and that the major products of this reaction are C-CAM-L dimers lends support to this notion.

Tissue transglutaminase has been implicated in the process of apoptosis and large protein species, formed by transglutaminase-catalysed cross-linking, have been identified in rat liver [16,22]. However, unlike the C-CAM-L homo-dimers identified here, these aggregates have apparent molecular masses greater than 5×10^3 kDa, contain multiple protein constituents, and appear to represent the highly cross-linked envelopes of cells undergoing programmed cell death [22].

Dimer formation is not a prerequisite for cell adhesion, suggesting that dimers may instead be involved in C-CAM-mediated signalling [8]. The finding that human C-CAM-L can bind protein tyrosine kinases of the src family [3] and that mouse C-CAM-L binds the protein tyrosine phosphatases SHP-1 and SHP-2 [1,5] led us to propose a model in which adhesion and signalling are regulated by the C-CAM monomer/dimer ratio within cells [1,23]. In the monomeric state, C-CAM-L may more readily bind tyrosine kinases, leading to the generation of a positive growth signal. SHP-1 and SHP-2, which have two phosphotyrosine-binding SH2 domains, are suggested to bind to C-CAM-L homo-dimers, which might lead to termination of the positive growth signal. C-CAM-L dimers, covalently cross-linked by transglutaminase, would, according to this model, be predicted to be locked into a form which suppresses cellular growth. It is interesting to note therefore, that when transfected into highly tumorigenic cells, both rC-CAM-L [7] and mC-CAM-L [6], but not their short cytoplasmic domain counterparts, act as suppressors of tumour growth. An intriguing and testable hypothesis, suggested by our current studies, is that these long cytoplasmic domain isoforms may be covalently modified by tissue transglutaminase, leading to increased stabilisation of C-CAM-L dimers and a potentiation of a negative growth signal. Such a role for tissue transglutaminase is in agreement with observations in other laboratories that increased transglutaminase activity modifies cancer cells [24] and has negative effects on cellular growth [17].

The precise role of C-CAM dimer formation and transglutaminase stabilisation of C-CAM-L homo-dimers remains to be elucidated. However, the conservation of the two immediately adjacent glutamine residues in rat, mouse and human C-CAM-L and our demonstration that both rat and mouse C-CAM-L can act as transglutaminase substrates strongly suggests that transglutaminase-catalysed modification is likely to be a physiologically important modulator of C-CAM function.

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References

[1] Öbrink, B. (1997) Curr. Opin. Cell Biol. 9, 616–626.
[2] Olsson, H., Wikström, K., Kjellström, G. and Öbrink, B. (1995) FEBS Lett. 365, 51–56.
[3] Brümmer, J., Neumaier, M., Göpfert, C. and Wagener, C. (1995) Oncogene 11, 1649–1655.
[4] Afar, D.E.H., Stanners, C.P. and Bell, J.C. (1992) Biochim. Biophys. Acta 1134, 46–52.

[5] Beauchemin, N., Kunath, T., Robitaille, J., Chow, B., Turbide, C., Daniels, E. and Veillette, A. (1997) Oncogene 14, 783–790.
[6] Kunath, T., Ordonez-Garcia, C., Turbide, C. and Beauchemin, N. (1995) Oncogene 11, 2375–2382.
[7] Hsieh, J.T., Luo, W., Song, W., Wang, Y., Kleinerman, D.I., Van, N.T. and Lin, S.H. (1995) Cancer Res. 55, 190–197.
[8] Hunter, I., Sawa, H., Edlund, M. and Öbrink, B. (1996) Biochem. J. 320, 847–853.
[9] Baum, O., Reutter, W., Flanagan, D., Callanan, H., Lim, Y.P., Lin, S.H. and Hixson, D.C. (1995) Eur. J. Biochem. 228, 316–322.
[10] Greenberg, C.S., Birckbichler, P.J. and Rice, R.H. (1991) FASEB J. 5, 3071–3077.
[11] Lorand, L., Losowsky, M.S. and Miloszewski, K.J.M. (1980) Prog. Hemost. Thromb. 5, 245–290.
[12] Rothnagel, J.A. and Rogers, G.E. (1984) Mol. Cell. Biochem. 58, 113–119.
[13] Kinsella, M.G. and Wight, T.N. (1990) J. Biol. Chem. 265, 17891–17898.
[14] Aeschlimann, D. and Paulsson, M. (1991) J. Biol. Chem. 266, 15308–15317.
[15] Hohenadl, C., Mann, K., Mayer, U., Timpl, R., Paulsson, M. and Aeschlimann, D. (1995) J. Biol. Chem. 270, 23415–23420.
[16] Fesus, L., Thomazy, V., Autuori, F., Cerù, M.P., Tarcsa, E. and Piacentini, M. (1989) FEBS Lett. 245, 150–154.
[17] Katoh, S., Nakagawa, N., Yano, Y., Satoh, K., Kohno, H., Ohkubo, Y., Suzuki, T. and Kitani, K. (1996) Exp. Cell Res. 222, 255–261.
[18] Ando, Y., Imamura, S., Owada, M.K. and Kannagi, R. (1991) J. Biol. Chem. 266, 1101–1108.
[19] Grootjans, J.J., Groenen, P.J.T.A. and de Jong, W.W. (1995) J. Biol. Chem. 270, 22855–22858.
[20] Laemmli, U.K. (1970) Nature 227, 680–685.
[21] Murray, A.J., Lewis, S.J., Barclay, A.N. and Brady, R.L. (1995) Proc. Natl. Acad. Sci. USA 92, 7337–7341.
[22] Knight, C.R.L., Hand, D., Piacentini, M. and Griffin, M. (1993) Eur. J. Cell Biol. 60, 210–217.
[23] Öbrink, B. and Hunter, I. (1998) in: Cell Adhesion and Communication Mediated by the CEA Family: Basic and Clinical Perspectives (Stanners, C.P., Ed.), Harwood Academic, Amsterdam (in press).
[24] Beninati, S. (1995) Cancer J. 8, 234–235.