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Recombinant Soluble Human CD69 Dimer Produced in *Escherichia coli:* Reevaluation of Saccharide Binding[1]

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We reevaluate here an earlier report of monosaccharide binding by the C-type lectin-like, leukocyte surface protein CD69 in the form of a recombinant soluble dimer, and we examine polysaccharide binding by the protein. We have expressed in *Escherichia coli* a new construct of the extracellular part (Q_{65}-K_{199}) of human CD69. We describe the folding *in vitro* to produce, in good yield, the protein in a soluble, disulphide-linked, dimeric form, and the results of binding experiments with monosaccharides: glucose, galactose, mannose, fucose, *N*-acetylglucosamine, and *N*-acetylgalactosamine, linked to bovine serum albumin. Monosaccharide-binding signals are not detectable. Among the polysaccharides, heparin, chondroitin sulphates A, B, and C, fucoidan, and dextran sulphate, CD69 dimer gives a weak binding signal with fucoidan.

CD69 is an inducible cell surface glycoprotein appearing early upon activation of leukocytes, and has been shown to act as a signal transmitting receptor (1–3). The extracellular domain of 135 amino acids contains a C-type lectin-like domain (4–6). In an earlier report (7), binding to *N*-acetylglucosamine linked to bovine serum albumin, and inhibition of the binding by *N*-acetylglucosamine and *N*-acetylgalactosamine were described for a recombinant dimeric form of human CD69 produced in *E. coli* and folded *in vitro.* Attempts to generate further supplies of this protein construct have proven unsuccessful.[4] We have now expressed in *E. coli* and refolded, *in vitro,* a new construct of the extracellular part of CD69, and report here that, as opposed to the previous publication, the folded dimeric protein elicits no binding signals with monosaccharide derivatives.

MATERIALS AND METHODS

Protein expression and isolation. The cDNA encoding the extracellular part, residues Q_{65}-K_{199}, of human CD69 was amplified by PCR (primer sequence, forward 5′ CGG AAT TCC AAT ACA ATT GTC CAG GCC AAT; reverse 5′ CCC AAG CTT TTA TTT GTA AGG TTT GTT ACA) from the full length cDNA (gift from Dr Francisco Sanchez-Madrid, Servicio de Immunologica, Spain) and cloned into the EcoRI/Hind III sites of pET28a(+) (Novagen, Madison, Wisconsin). The protein tagged with a hexa-His sequence and T7 epitope (Fig. 1), designated rCD69^t, was expressed in inclusion bodies in *Escherichia coli* BL-21. The inclusion bodies were isolated by centrifugation (10,000 g) of sonicated bacteria and solubilized in 500 mM NaCl containing 20 mM sodium phosphate buffer pH 7.8, 10 mM Tris buffer pH 8.0, 8 M urea and 5 mM 2-mercaptoethanol. The recombinant protein was recovered by affinity chromatography using Ni-NTA Superflow resin (Qiagen, Crawley, UK) and low pH elution according to the manufacturer's recommendations. The eluate was adjusted to pH 6.0 with NaOH and stored at −70°C in aliquots containing 10 mg protein at an approximate concentration of 1 mg/ml, as assayed by the Coomassie Blue Plus reagent (Pierce & Warriner, Chester, UK), using bovine serum albumin as a standard. The yield of protein was approximately 30 mg per liter of culture.

Protein folding and isolation of soluble dimeric protein. Folding was typically of a 10 mg aliquot: 2-Mercaptoethanol was added to a final concentration of 10 mM, and the reduced protein solution was added dropwise (approximately 1 ml/min) to 300 ml of a constantly swirled solution containing 50 mM Tris buffer pH 8.25, 2 M urea, 2 mM $CaCl_2$, 1.34 mM reduced glutathione and 0.67 mM oxidized glutathione. This had been filtered through a 0.2 μ filter, degassed using a vacuum pump, and then bubbled with Ar for 5 min immediately prior to use. The protein solution was immediately loaded at a flow rate of 1.5 ml/min onto a Ni-NTA Superflow column (2 ml bed vol., 0.7 cm diameter) which had been equilibrated in the folding solution. In preliminary experiments, it was found that after the dilution stage, concentration of the protein on the nickel affinity column substantially increased the yield of dimeric protein. The column was washed with 10 column volumes of folding solution minus glutathione followed by 50 mM Tris buffer pH 8.0, and eluted with 50 mM Tris buffer pH 8.0 containing 50 mM EDTA. Eluted protein, monitored by Coomassie Blue Plus reagent, was pooled,

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[4] K. Bezouska, personal communication.

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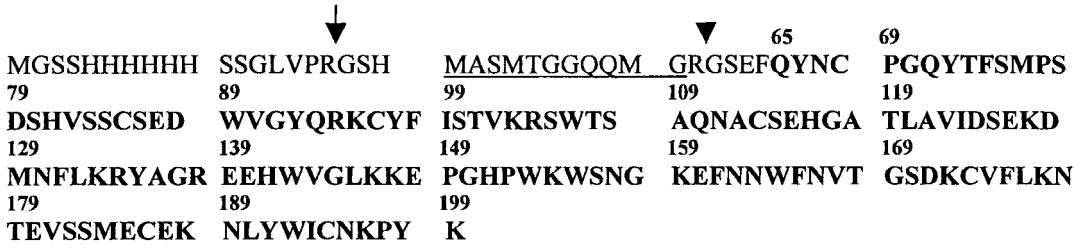

FIG. 1. Amino acid sequence of the recombinant protein construct designated rCD69^t. The construct contained the extracellular part of human CD69, Q_{65}-K_{199} (bold) fused at the N-terminus to a 36-amino-acid tag that contains a hexa-His sequence, and the T7 epitope (underlined). The symbols (▼) and (↓) indicate predicted cleavage sites on the tag sequence for trypsin and for both trypsin and thrombin, respectively. The numbering system for CD69 relates to that of the full length protein (6).

centrifuged at 2000 g for 5 min, and dialysed at 4°C for 24 h against 50 mM Tris buffer pH 8.0, followed by 20 mM PIPES buffer pH 7.5 for a further 24 h. The protein solution was centrifuged at 2000 g for 10 min and the supernatant concentrated to about 8 mg/ml, by ultrafiltration using a PM10 membrane (Amicon, Beverly, MA). The yield of soluble protein was approximately 3.5 mg per 10 mg of starting, unfolded protein. The folded protein was chromatographed by FPLC using a Superdex 75 16/30 column equilibrated in 150 mM NaCl containing 20 mM PIPES buffer pH 7.5 (PIPES/NaCl). Fractions within the major $UV_{280\,nm}$ adsorbance peak were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 4–12% Bis-Tris gels (Novex, San Diego, CA) stained with Coomassie Blue reagent; fractions containing reducible dimeric protein were retained and stored in aliquots at −70°C. Attempts at refolding by a dialysis method as described previously (7) yielded no soluble protein.

For removal of the hexa-His-Tag and the T7 epitope, to yield rCD69, the dimeric rCD69^t in PIPES/NaCl was incubated at 20°C for 20 min with trypsin (sequencing grade, Sigma, Poole, UK) at a protein to trypsin ratio of 100:1. Following incubation, phenylmethylsulphonyl fluoride and *N*-p-tosyl-L-lysine chloromethyl ketone (Sigma), were each added to a final concentration of 2 mM. The digest was chromatographed on a Sephacryl S100 HR 16/60 column (Amersham Pharmacia Biotech, St Albans, UK) equilibrated in 0.2 M NaCl. Fractions were analysed by SDS–polyacrylamide gel electrophoresis using 10–20% Tricine gels (Novex).

Antigenic analyses. The following murine monoclonal antibodies were used: antibodies to CD69 (gifts of Dr. Francisco Sanchez-Madrid) were culture supernatants used at 1:2 dilution, CH/1, CH/4, FAB-1 and TP/8 are conformation-dependent and bind to the native CD69 on activated peripheral blood lymphocytes, and CH/2 binds to denatured CD69 polypeptide (8). Antibody 3.2.3. (9) directed to a conformational epitope on the rat NK cell receptor NKR-P1 (gift of Dr. William H. Chambers) was used as ascites at a dilution of 1:500. The antibodies to the T7 epitope (Novagen) and to the poly-His tag (Sigma) were used at 2 μg/ml. For binding experiments, 1.5 μg of recombinant protein, in 50 μl of 20 mM phosphate-buffered saline pH 7 (PBS), was added to duplicate wells (Falcon 3912) and incubated at 37°C for 1 h. Wells were washed and incubated with Blocker Casein (Pierce) for 1 h at room temperature. After three washes with PBS, wells were incubated for 1 h with 50 μl of the murine antibodies, washed and incubated with 50 μl of 1:500 dilution of goat anti-mouse immunoglobulins conjugated with horseradish peroxidase (DAKO, Glostrup, Denmark). Binding was detected using O-phenyldiamine substrate, and absorbance was read at 490 nm. In one experiment, the protein (30 μg/ml) was reduced in the presence of 10 mM dithiothreitol for 30 min, alkylated by the addition of iodoacetamide at 11 mM for a further 30 min and heated at 100°C for 5 min before coating onto microwells as described above.

For antigenic analysis of the recombinant proteins following SDS–polyacrylamide gel electrophoresis, the proteins were separated in 10–20% Tricine gels and electrotransferred onto nitrocellulose under standard conditions. Lanes were stained with Ponceau S to reveal protein; duplicate lanes were incubated in Blocker Casein solution for 30 min, followed by monoclonal antibody to T7 epitope (5 μg/ml), for 30 min. The blots were washed and incubated for 30 min with a 1:200 dilution of rProtein LA conjugated to horseradish peroxidase (Actigen, Cambridge, UK). After washing, the bound peroxidase was revealed using 4-chloro-1-naphthol/3,3′-diaminobenzidine tetrahydrochloride substrate kit (CN/DAB, Pierce & Warriner).

Saccharide-binding studies with recombinant CD69 proteins. The recombinant proteins rCD69^t and rCD69 were radio-iodinated using $Na^{125}I$ and chloramine T under mild conditions (10); specific activities of the proteins were approximately 0.4 MBq per μg. Radioiodination of the proteins was confirmed by their co-migration in SDS–polyacrylamide gels with the unlabelled species (radiolabelled bands were visualized by autoradiography of dried gels). Monosaccharide binding was examined by using the following six monosaccharides linked to bovine serum albumin (gifts of Dr. Y. C. Lee): $fucose_{20}$, $galactose_{34}$, $glucose_{51}$, $mannose_{35}$, *N*-$acetylgalactosamine_{20}$ and *N*-$acetylglucosamine_{28}$, where the subscripts indicate the number of monosaccharides per mol of bovine serum albumin. Neoglycoproteins (1.5 μg per well) in 150 mM NaCl containing 10 mM Tris buffer, pH 7.4 (TBS) were dried at 37°C overnight onto the surface of microwells (Dynex, Billingshurst, UK). Wells were blocked using Blocker casein solution for 1 h, washed with TBS and varying amounts of ^{125}I-labelled rCD69^t added (10^4–2×10^5 cpm per well) for 2 h. Wells were washed, and bound radioactivity was measured using a gamma counter. Binding of ^{125}I-labelled rCD69^t (5×10^5 cpm per well) to the polysaccharides, heparin, chondroitin sulphates A, B and C, fucoidan and dextran sulphate (Sigma), was determined as described above, except that the microwells were pre-coated for 1 h with poly-L-lysine (poly-L-lysine hydrobromide, DP1426, average molecular mass 298000, cat no. P1399, Sigma), 2.5 μg per well, washed, and varying amounts of the polysaccharides (0.15–5 μg per well) were added and incubated at 37°C overnight. To examine the influence of pH on binding of rCD69^t and rCD69 to fucoidan, varying amounts of fucoidan (1.5–50 μg per well) were dried at 37°C onto microwells, blocked with Blocker casein and ^{125}I-labelled proteins were added (9×10^4 cpm per well) in PBS at pH 5, 6, 7 or 8 containing 0.1% casein.

RESULTS

Preparation of the recombinant, soluble CD69 dimer tagged with T7 epitope and hexa-His sequence, rCD69^t. Induction of protein production in transformed *E. coli* in the presence of 1 mM isopropyl β-D-thiogalactoside (IPTG) at 30°C for 2 h, is shown in Fig. 2A (lane 2), and the denatured protein purified from solubilized inclusion bodies, in lane 3. After folding and gel filtration on Superdex 75, 80–90% of the soluble protein behaved (as assessed by SDS–PAGE) as a disulphide-linked

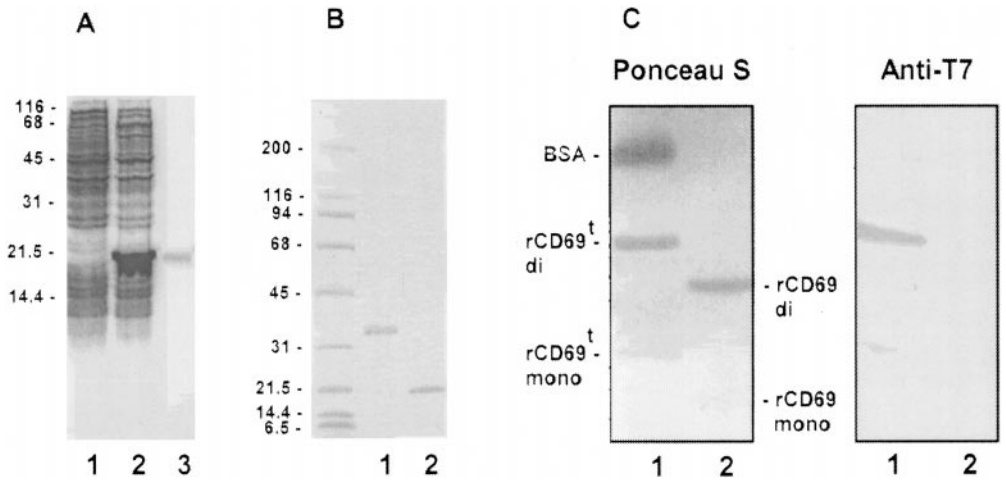

FIG. 2. SDS–polyacrylamide gel electrophoretic analyses of rCD69^t and and rCD69. (A) Electrophoresis was carried out on bacterial lysates (cells from 0.5 ml of culture), in a 15% polyacrylamide gel, in the presence of reducing agent, before (lane 1) and after (lane 2) induction with IPTG, and on the rCD69^t (lane 3) isolated from solubilized inclusion bodies. The gel was stained with Coomassie blue; molecular weight markers ($\times 10^{-3}$) are shown. (B) 1 μg of purified, folded, dimeric protein, rCD69^t, was electrophoresed in a 4–15% gradient gel in the absence (lane 1) and presence (lane 2) of reducing agent. The gel was stained with Coomassie blue. Molecular weight markers ($\times 10^{-3}$) are shown. (C) rCD69t (lanes 1) and the product (rCD69) obtained after limited trypsin digestion (lanes 2) were electrophoresed on a 10–20% polyacrylamide gel and electroblotted onto nitrocellulose. One blot was stained with Ponceau S to reveal proteins. A duplicate blot was overlaid with anti-T7 followed by rProtein LA-HRP and CN/DAB detection system to reveal immunoreactivity in the undigested protein, and the lack of immunoreactivity after the partial digestion. Bovine serum albumin (BSA) was added to rCD69^t to act as a negative control for anti-T7 reactivity. A small amount of monomer is present in the dimer preparation.

dimer of apparent molecular mass 35 kDa before reduction, and 20 kDa after reduction (Fig. 2B). The mass of the soluble, dimeric protein, rCD69^t, recorded by electrospray mass spectrometry (ES-MS) was 38,827 Da (results will be described in detail elsewhere). This corresponds to the calculated mass of the dimeric tagged protein, 39087.7 Da, less the two terminal methionines often cleaved in proteins expressed in *E. coli* (11).

Preparation of the recombinant, soluble CD69 dimer with the hexa-His and T7 epitope sequences removed rCD69. The 36 amino acid tag sequence contains two potential trypsin cleavage sites (Fig. 1). In exploratory studies with the refolded rCD69^t, we observed that cleavage readily occurs at 20°C at low trypsin concentrations. Cleavage was monitored by loss of reactivity with T7 antibody and change in mobility of the cleaved protein as revealed by Ponceau S staining of blotted protein (Fig. 2C). After 15 min digestion at a protein to trypsin ratio of 100:1, a stable fragment was produced, designated rCD69, migrating at about 30 kDa. The mass determined by ES-MS for rCD69 was 32,287.3 Da in close agreement with the calculated mass, 32,288.1 Da, for the CD69 sequence Q_{65}-K_{199} plus the residual four amino acids GSEF of the tag.

Immunoreactivities of rCD69^t and rCD69. The lack of the hexa-His tag and the T7 epitope on rCD69 was corroborated by the lack of binding of anti-poly-His and anti-T7 antibodies to rCD69 in contrast to their binding to rCD69^t (Fig. 3). However, the immunoreactivities of rCD69^t and rCD69 with the antibodies to CD69 were similar. Both proteins were bound by the four antibodies CH/1, CH/4, FAB-1 and TP/8 directed to conformational antigens on CD69, but not by antibody 3.2.3. directed to a conformational antigen on rat

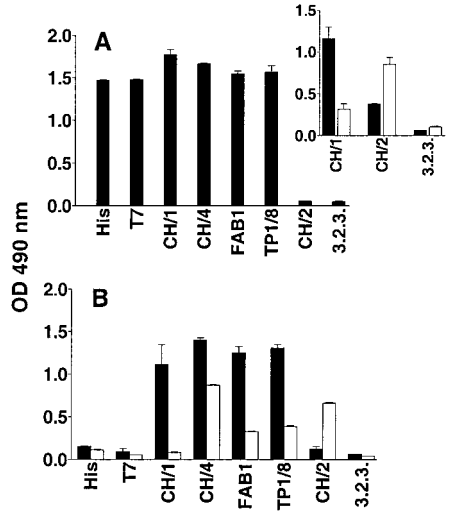

FIG. 3. Immunoreactivities of rCD69^t and rCD69. (A) rCD69^t and (B) rCD69 were coated onto microwells before (solid bars) and after reduction and heat denaturation (open bars) and the binding of antibodies detected as described under Materials and Methods. Results are expressed as means of duplicate wells with the range indicated by error bars.

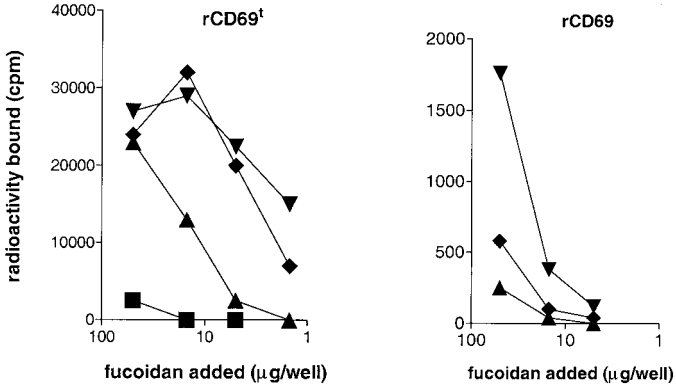

FIG. 4. Binding of radio-iodinated rCD69^t and rCD69 to fucoidan at different pH values. Varying amounts of fucoidan were coated onto microwells and probed with radio-iodinated rCD69^t and rCD69 proteins at pH 5 (▼), pH 6 (◆), pH 7 (▲), and pH 8 (■), as described under Materials and Methods.

NKR-P1 (Fig. 3). There was little or no binding by antibody CH/2 which recognizes the denatured CD69, unless the proteins were reduced and denatured (Fig. 3A, inset, and Fig. 3B); the binding signals with the conformation-sensitive anti-CD69 antibodies were diminished when the two CD69 proteins were denatured.

Saccharide-binding studies with rCD69^t and rCD69 proteins. No binding signals were detected for rCD69^t with any of the monosaccharides (results not shown). Binding of rCD69^t was observed, however, to the sulphated polysaccharide fucoidan (Fig. 4), but not to the proteoglycans, heparin, chondroitin sulphates A, B, and C, and dextran sulphate (results not shown).

Knowing that the pK_a of the imidazole of histidine is 6.04, we considered the possibility that the hexa-His sequence on rCD69^t, by functioning as a polycation, would contribute an increased positive charge to the protein upon protonation at a pH of 6 and below, and bind more strongly to fucoidan. This was corroborated in binding experiments with rCD69^t and rCD69 (Fig. 4). The binding of rCD69^t to fucoidan was greatest at pH 5 and 6; it was considerably lower at pH 7 and minimal at pH 8. With rCD69 which lacks the hexa-His sequence, the binding signal with fucoidan was low (maximal signal about 20-fold lower), although still pH-dependent.

DISCUSSION

A recombinant form of human CD69 was designed to include the extracellular part, residues 65–199, of the native protein linked to a 36 amino acid tag at the N-terminus containing a hexa-His sequence and T7 epitope for purification and immuno-detection purposes. This region of CD69 contains seven cysteines including Cys_{68} which, in detailed studies to be published elsewhere, we have shown to form an interchain disulphide bond, giving rise to a homodimer analogous to the natural form of the extracellular part of the membrane-associated CD69. Considerable efforts were expended to produce well-folded, dimeric rCD69 for reevaluating the monosaccharide binding, as the affinity of binding of monomeric CD69 to ligand may be very low. By analogy with the selectins (12, 13) and pulmonary surfactant protein A (14), however, even higher oligomers would be required for a full evaluation of the ligands.

In initial folding experiments, dilution of denatured rCD69^t into the redox-containing renaturant solution followed by dialysis over several days into Tris buffer, and ultrafiltration to concentrate the protein, yielded predominantly soluble, monomeric protein. It was reasoned that immediate concentration of the protein on a nickel column, while in the renaturant solution, might promote formation of the interchain disulphide bond, at the same time as allowing shuffling of disulphides to continue, forming the intrachain disulphide bonds as found on the natural protein. There is the additional benefit of the promotional effect of heavy metals on disulphide bond formation (15). Good yields of the soluble dimeric protein were obtained by this procedure. The immunoreactivities of rCD69^t and rCD69 with conformation-dependent antibodies raised to natural CD69, the presence of a relatively trypsin-resistant domain, and the inter- and intra-chain disulphide bonds revealed by mass spectrometry of tryptic peptides (to be described in detail elsewhere) all point to the correct folding of the recombinant protein.

The ligands for CD69 are not yet known. The deletions in loop regions of the CRD and the lack of conservation of amino acids that bind calcium through which monosaccharides can be ligated (16, 17), suggest that the mechanisms of ligand binding may differ from those for the collectins and the selectins. The CD69 dimer differs from the collectins also in its lack of monosaccharide-binding (oligomeric forms of the protein are yet to be evaluated), but the lack of monosaccharide binding is a feature shared with the selectins; these require oligosaccharide sequences for eliciting binding signals (18). Our initial examination of polysaccharide-binding by the hexa-His-tagged form of CD69 revealed substantial binding to fucoidan. Lowering the pH increased the binding. Most likely, the hexa-His sequence thus protonated acts as a polycation that binds to the negatively charged sulphate groups on fucoidan. This is an important consideration in interpreting binding data with recombinant proteins containing a poly-His sequence. The biological significance of the weak, pH-dependent fucoidan-binding by rCD69 dimer is under investigation to determine whether it is a reflection of traces of the His-tagged form, or related to fucoidan binding by the NK-cell lectins Ly49A and C (19–21) and L-selectin (22, 23).

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