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CD 69 ANTIGEN OF HUMAN LYMPHOCYTES IS A CALCIUM-DEPENDENT CARBOHYDRATE-BINDING PROTEIN

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CD69 is a signal transducing molecule of hematopoietic cells. Previous molecular cloning of CD69 has revealed a type II transmembrane orientation and the presence of an extracellular domain related to the Ca^{2+}-dependent (C-type) animal lectins. As the predicted amino acid sequence for the lectin-like domain is highly divergent from those of other C-type lectin-like proteins - a feature shared with NKR-P1 of natural killer cells - CD69 and NKR-P1 are among proteins assigned to a separate group, group V. To initiate ligand identification studies, we have prepared soluble forms of CD69 protein by bacterial expression of its extracellular portion. We show that cysteine 68 located in the short membrane-proximal neck region of CD69 which adjoins the C-terminal lectin-like domain is a critical element for dimerization. We have evidence that the soluble dimeric CD69 has a tight association with calcium, a feature shared with NKR-P1, and that it is a carbohydrate-binding protein with N-acetyl-D-glucosamine and N-acetyl-D-galactosamine as the best inhibitors: 4-8 x 10^{-5} M giving 50 % inhibition of binding to N-acetyl-D-glucosamine neoglycoprotein. Thus, the tight association with calcium and high affinities for carbohydrate binding appear to be features of at least two members of the C-type lectin group V.

CD69 (1,2) is a signal transducing molecule of leukocytes that exists as a dimeric disulfide-linked membrane glycoprotein with subunits in the range of 26 - 34 kDa (3-5). It is constitutively expressed on $CD3^{bright}$ thymocytes (6) and on platelets (7) and its expression is induced upon lymphocyte activation (8). Molecular cloning has revealed that CD69 is a type II transmembrane

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Abbreviations: BSA, bovine serum albumin; TBS, 10 mM Tris HCl, pH8, with 150 mM NaCl; TBS+C, TBS containing 10mM $CaCl_2$; TBS+E, TBS containing 10mM EGTA.

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protein with a predicted molecular mass 22,559 daltons and a carboxy-terminal region that resembles the carbohydrate-recognition domains of Ca^{2+}-dependent (C-type) animal lectins (9-11). Dimeric proteins of this type have been assigned as group V among the C-type lectin family (12), and they include the NK cell-associated proteins such as NKR-P1, Ly-49 and NKG2 (13,14). CD69 differs from these latter glycoproteins in being shorter: it lacks a "stalk" region between the transmembrane domain and the "neck" region of the lectin-like domain (15). Moreover, analysis of the gene for human CD69 has revealed a single copy (15), which contrasts with the gene families that exist for NKR-P1 and Ly-49 (14). The aim of the present study has been to produce recombinant soluble forms of CD69 in *Escherichia coli*, and to determine whether CD69 is a carbohydrate-binding protein.

MATERIALS AND METHODS

DNA restriction and modification enzymes, linkers, pMAL primer, factor Xa, and protein fusion and purification system were from New England Biolabs, Beverley, MA, U.S.A. Neoglycoproteins of $GlcNAc_{23}$-BSA, Fuc_{23}-BSA, Man_{23}-BSA, Gal_{23}-BSA and Glc_{23}-BSA were kindly provided by Prof. Y.C.Lee, Johns Hopkins University, Baltimore, MD, U.S.A. ($GalNAc_{23}$-BSA was not available for testing). Monoclonal antibodies against CD69, BL-KFB/B1 and BL-Ac/p26 (9) were used as ascitic fluid, 1:4000 dilution. Other laboratory chemicals and Sephacryl S-200 SF were from Sigma, St.Louis, MO, U.S.A.

Production of soluble forms of CD69 in *E.coli*. Three proteins containing extracellular portions of CD69 antigen (Fig.1a) were prepared (Fig.1b): CDA-401 encompasses the extracellular portion minus two amino acids starting with the glycine 64; CDA-411 is further shortened and the cysteine 68 is substituted by arginine; CDA-421 consists of the lectin-like domain. The corresponding DNA fragments were transferred from cDNA clone CD69.10 (9) into pMALc2 expression vector downstream from a unique EcoRI site; EcoRI linkers were used to correct the reading frames, and the expression plasmids were sequenced from double-stranded templates using pMAL primer and TaqTrack sequencing protocol (Promega, Madison, WI, U.S.A.). Soluble

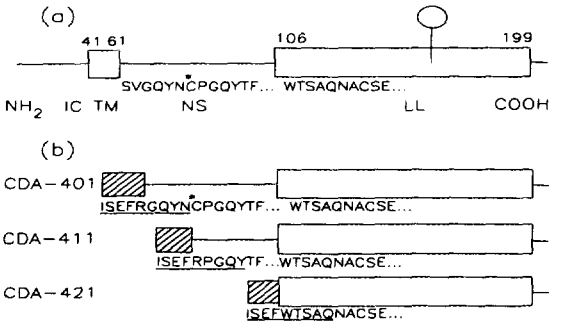


Fig.1. Schematic representations of the full-length CD69 and of truncated recombinant soluble forms. In (a), the full length CD69 monomer is depicted; the N-terminal end (NH_2) is intracellular (IC); between the transmembrane (TM) domain and the lectin-like (LL) domain at the C-terminal (COOH), extracellular part of the molecule is a short neck sequence (NS), and portions of amino acid sequence are displayed in a single-letter amino acid code; O— indicates a potential glycosylation site (ref. 9-11). In (b), modified soluble forms of CD69 produced by bacterial expression are presented. These include four to five additional amino acids (hatched blocks) introduced from the expression constructs. N-terminal amino acid sequences confirmed by Edman degradation are underlined. Cysteine 68 which in the present study is found to be involved in dimerization is asterisked.

CD69 proteins were expressed as fusion proteins with the bacterial maltose-binding protein in *E.coli* strain NM522 and cleaved with factor Xa essentially as described previously (16). After cleavage and purification under denaturing conditions, soluble CD69 proteins were renatured (16) while monitoring the appearance of CD69 epitopes recognized by the two monoclonal antibodies. Ten cycles of Edman degradation (protein sequenator 470A, Applied Biosystems, Palo Alto, CA) were performed with the cleaved proteins [which contained an additional 4-5 amino acids from the expression vector (Fig.1b)], to establish the presence of the correct amino acid sequence.

Gel filtration analyses. A column of Sephacryl S-200 SF (1x150 cm) was equilibrated and eluted in TBS+C. Approximately 10 mg of soluble CD69 proteins were applied in 1 ml of TBS+C, the elution profiles monitored at 280 nm and the peaks analysed by SDS-polyacrylamide gel electrophoresis (17).

Carbohydrate-binding assays. Dimeric CDA-401 and monomeric CDA-421 proteins were radioiodinated (16): specific activities $10x10^6$ and $4x10^6$ cpm/mg, respectively. Binding of these proteins to neoglycoproteins (1 μg each) which had been electrophoresed and transferred onto nitrocellulose membrane or immobilized on plastic microtiter wells were assayed as described previously (16).

Decalcification and recalcification experiments. Radiolabeled CDA-401 protein (10^6 cpm) was mixed with the unlabelled protein (10 μg) in TBS+C with 5% BSA and dialysed in duplicate bags for 2 days against TBS+E or 0.1 M Tris-HCl buffer pH 10. One of each duplicate aliquot was dialysed for a further 2 days against TBS+C, and the other aliquot against TBS+E, and used in binding experiments with GlcNAc-BSA in microtiter wells (16).

RESULTS

The elution profiles of the three recombinant soluble proteins, CDA-401, CDA-411 and CDA-421, subjected to gel filtration are shown in Fig.2. CDA-401, which represents the extracellular portions of CD69 starting with glycine 64 (Fig.1), eluted as two peaks of almost equal ratio with apparent molecular masses of 35 and 17 kDa, corresponding to dimeric and monomeric arrangements of the predicted 17 kDa subunits, respectively. In accord with this assignment the protein in the former peak migrated in the range of 32-34 kDa when analysed by SDS polyacrylamide electrophoresis under nonreducing conditions (Fig.3, lane 1). Under reducing conditions it migrated as a monodisperse band with an apparent molecular mass of 17 kDa (Fig.3, lane 4). CDA-411, which had a shortened neck region and a cysteine to arginine mutation at amino acid 68 (Fig.1b), eluted as two peaks corresponding to dimers and monomers in the ratio 1 : 4. Analysis of the former peak by SDS electrophoresis revealed a monodisperse band with an apparent molecular mass of 17 kDa under both nonreducing and reducing conditions (Fig.3 lanes 2 and 5) showing that the dimerization is non-covalent. An even greater proportion of the peak chromatographing as a monomer was obtained for CDA-421, which contained exclusively the lectin-like domain, and analysis of the corresponding peak by SDS polyacrylamide gel electrophoresis showed a monodisperse band with an apparent molecular mass of approximately 14 kDa under both nonreducing and reducing conditions (Fig.3, lanes 3 and 6).

When the dimeric form of CDA-401 and the monomeric form of CDA-421 were radioiodinated and tested for binding to the BSA derivatized with GlcNAc, Fuc, Man, Gal or Glc on nitrocellulose membrane, binding of CDA-401 (but not CDA-421) was detected only to

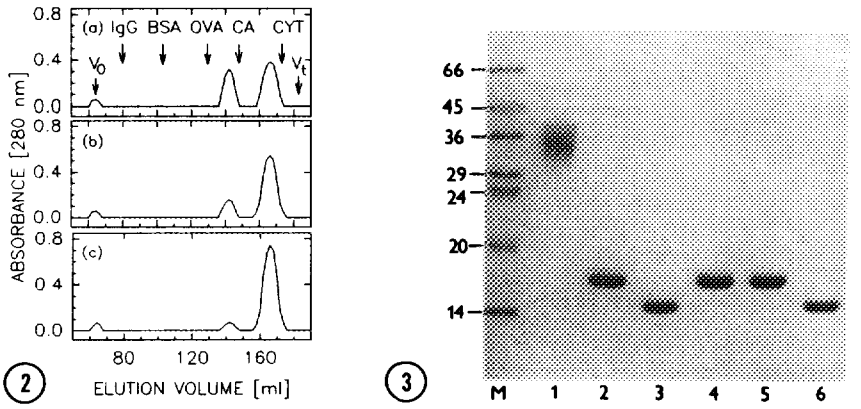
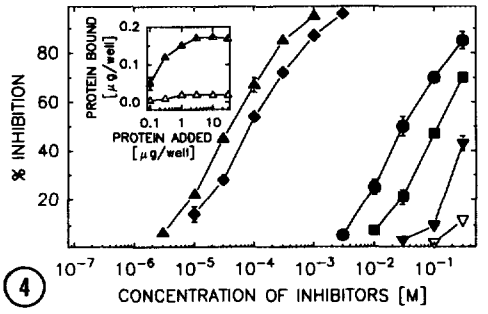


Fig.2. Analysis of soluble recombinant forms of CD69 by gel filtration. Approximately 10 mg of refolded soluble CD69 proteins were analysed on Sephacryl S-200 SF (1x150 cm) column as described under Materials and Methods. Panel (a), CDA-401; panel (b), CDA-411; panel (c), CDA-421. Elution positions of protein markers used to calibrate the column [immunoglobulin G (IgG), 150 kDa; bovine serum albumin (BSA), 65 kDa; ovalbumin (OVA), 44 kDa; carbonic anhydrase (CA),30 kDa; and cytochrome c (CYT), 12 kDa] are indicated together with the void (V_0) and total (V_t) volumes of the column.

Fig.3. Analysis of soluble CD69 proteins by SDS-polyacrylamide gel electrophoresis after gel filtration. Protein samples were boiled in sample buffer without (lanes 1-3) or with (lanes 4-6) reducing agent, and 5 µg of each sample was analysed on 15 % polyacrylamide gels as decribed under Materials and Methods. Molecular masses of marker proteins (M) are indicated in kDa. Lanes 1 and 4 contained CDA-401 peak eluting at 143 ml; lanes 2 and 5 CDA-411 peak eluting at 143 ml; lanes 3 and 6, CDA-421 peak eluting at 165 ml.

GlcNAc-BSA (not shown). Similarly, in microwell binding assays CDA-401 bound to GlcNAc-BSA in a saturable manner, while CDA-421 gave barely detectable binding (Fig.4, inset). When monosaccharides were used as inhibitors of CDA-401 binding to GlcNAc-BSA, N-acetyl-D-glucosamine and N-acetyl-D-galactosamine were the best inhibitors with IC_{50} values (concentrations giving 50 % inhibition of binding) of approximately 4-8x10^{-5} M. L-fucose and D-mannose were approximately one thousand times less active with IC_{50} of 3x10^{-2} and 10^{-1} M, respectively, while D-galactose and D-glucose gave negligible inhibition (Fig.4).

Binding of CDA-401 to GlcNAc-BSA immobilized on nitrocellulose was unaffected in the absence of external calcium and in buffer containing 10 mM EGTA, TBS+E (not shown). This suggested that binding is either calcium independent, or that calcium is tightly associated with the protein. To distinguish between these possibilities, we dialysed the radioiodinated protein against Tris buffer (lacking calcium) at pH 10. The protein was then dialysed against TBS+E or TBS+C, and the binding activities of these two preparations were compared with that of the control protein which had been dialysed against TBS+E (pH 8) followed by further dialyses against TBS+E or TBS+C. The dialysis against TBS+E alone did not impair the binding activity of CDA-401 towards GlcNAc-BSA (Fig.5a). However, dialysis against pH 10 buffer followed by dialysis against TBS+E (conditions that effectively decalcified the structurally related protein NKR-P1 - reference 16) resulted in a loss of carbohydrate-binding activity (Fig.5b); the binding activity was largely restored when the protein was recalcified by dialysis against TBS+C (Fig.5b).



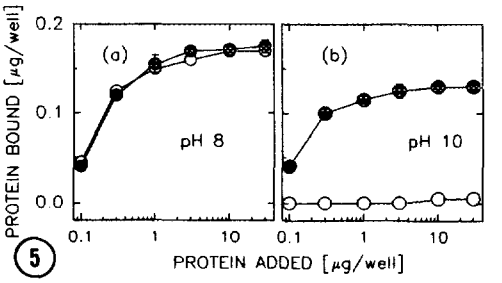


Fig.4. Quantitative assays of CDA-401 and CDA-421 binding to GlcNAc-BSA and inhibition of binding by monosaccharides. Inset shows binding of CDA-401 (closed symbols) and CDA-421 (open symbols) to microtiter wells coated with GlcNAc-BSA (1 μg added/well) determined as described under Materials and Methods. Main panel shows inhibition of binding of CDA-401 to GlcNAc-BSA coated microtiter wells (performed at half maximal binding) with monosaccharides. Symbols for the monosaccharides: ▲, N-acetyl-D-glucosamine; ♦, N-acetyl-D-galactosamine; ●, L-fucose; ■, D-mannose; ▼, D-galactose; ∇, D-glucose.

Fig.5. Influence of decalcification on carbohydrate binding by CDA-401. CDA-401 was dialysed in duplicate bags against TBS+E pH 8 (panel a) or Tris-HCl buffer, pH 10 (panel b); after the first dialysis, one of each duplicate bag was dialysed against TBS+C (closed symbols) and the other against TBS+E (open symbols), and the binding of these proteins to GlcNAc-BSA coated wells determined as described under Materials and Methods.

DISCUSSION

Collectively these results show that CD69 is a calcium-dependent carbohydrate-binding protein. This binding activity is detectable with the dimeric rather than the monomeric form of the soluble CD69 protein.

A comparison of the chromatographic and electrophoretic properties of the three recombinant proteins produced in this study indicates that dimerization is more efficient in CDA-401 which contains the neck region and cysteine 68 than in the truncated protein CDA-411 in which a part of the neck region is deleted and cysteine 68 is replaced by arginine. There is negligible dimerization in CDA-421 which contains the lectin-like domain only. Moreover, only in CDA-401 is the dimerization mediated by disulfide bonding, a feature of the native CD69 (ref 3). Even for CDA-401, however, the overall efficiency of dimerization is significantly lower than that observed previously for NKR-341, the recombinant soluble protein which contains the extracellular portion of rat NKR-P1 (ref 16). This lower efficiency of dimerization may thus be related to the absence of a stalk region and of three clustered C-terminal cysteines which are present in the latter protein.

Recognition of N-acetylglucosamine and N-acetylgalactosamine by CDA-401 is a feature shared with NKR-P1 of the rat (16). However, the specificities of the two proteins differ in that there is no detectable binding of CDA-401 to the immobilized fucose neoglycoprotein which is clearly bound by NKR-P1 (ref 16). Moreover, the IC_{50} values for the monosaccharides were substantially higher for CDA-401. For example, for CDA-401, IC_{50} values of 4-8 x 10^{-5} M have been calculated for N-acetylhexosamines compared with 10^{-7} M for NKR-P1. The lower affinity

of CD69 towards N-acetylglucosamine is also apparent from the lack of detectable binding of the monomeric protein, CDA-421 to immobilized GlcNAc-BSA.

Human CD69 and rat NKR-P1 studied previously (16, 18) are members of an expanding family of lectin-like proteins expressed at the surface of lymphocytes as type II transmembrane proteins that are disulphide-linked dimers. As the amino acid sequences of the lectin-like domains of these lymphocyte proteins are highly divergent from those of other C-type lectin-like proteins, they have been designated a separate group, group V (ref 12). This divergence with lack of one or more of the amino acids that ligate Ca^{2+} in the proteins that bind mannose or galactose investigated so far raised doubts that the group V proteins would bind saccharide ligands and Ca^{2+} (12). Three of these proteins have now been shown to bind carbohydrates: NKR-P1 (16, 18), Ly49A (19) and CD69 (this report). Our observations on NKR-P1 and CD69 suggest that the way these proteins ligate Ca^{2+} may be different from that seen for the structurally investigated mannan-binding protein (20). A striking feature of NKR-P1 and CD69 is their extremely tight association with Ca^{2+} such that it cannot be removed at physiological pH even after prolonged exposure to chelating agents. This high affinity Ca^{2+} binding seems to be paralleled in these proteins by relatively strong binding to carbohydrate as evidenced by the IC_{50} values for monosaccharides: 40 - 400 times lower for CD69 and 100,000 times lower for NKR-P1 that with those for the classical C-type lectins, the collectins (21). Our structural investigations underway are aimed at understanding these unusual features at the atomic level.

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