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The dimensions of the T lymphocyte glycoprotein leukosialin and identification of linear protein epitopes that can be modified by glycosylation

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Leukosialin (CD43) is a major glycoprotein of T lymphocytes whose extracellular domain of 224 amino acids contains on average one *O*-linked carbohydrate unit per three amino acids. This suggests an unfolded structure for the extracellular domain which has now been established to extend to a length of 45 nm by transmission electron microscopy following low angle rotary shadowing. The antigenicity of rat leukosialin has been studied using nine monoclonal antibodies (MAbs) whose binding is differentially affected by the cell type on which leukosialin is expressed and by the removal of sialic acid. From these observations it appears that the epitopes are affected by glycosylation, yet seven of the nine MAbs reacted clearly with the extracellular domain of leukosialian expressed in an unglycosylated form in *Escherichia coli*. The MAbs showing this positive reaction included three of the four antibodies whose epitopes were affected by neuraminidase treatment of leukosialin. It thus appears that linear protein epitopes are recognized and that some of these can be modified in the native structure by glycosylation. The positions of the antigenic determinants have been mapped by expressing fusion proteins of different lengths and the identity of one epitope was proven by the binding of two MAbs to an octapeptide expressed as a fusion protein. For three MAbs, the location of epitopes in the native protein was confirmed by electron microscopy of shadowed leukosialin-Fab complexes. Overall it is concluded that leukosialin is a major component at the periphery of the T lymphocyte and that despite its high level of glycosylation, protein determinants are exposed that could be ligands in cell interactions.

Key words: CD43/electron microscopy/epitopes/glycoprotein/mucin

Introduction

Leukosialin (sialophorin, CD43) is a major cell-surface sialoglycoprotein of thymocytes and T lymphocytes which is not expressed on resting B lymphocytes (Gahmberg *et al.*, 1976; Williams *et al.*, 1977; Brown *et al.*, 1981; Remold-O'Donnell *et al.*, 1987). It is also found on neutrophils, bone marrow stem cells and B-lineage cells after activation (Dyer and Hunt, 1981; Gulley *et al.*, 1988; Wiken *et al.*, 1988; Stross *et al.*, 1989). Leukosialin consists of an extracellular portion of 224, 229 or 234 amino acids in rat (Killeen *et al.*, 1987), mouse (Cyster *et al.*, 1990) and man (Pallant *et al.*, 1989; Shelley *et al.*, 1989) respectively, and attached to this there are 70-85 *O*-linked carbohydrate chains but few or no *N*-linked structures. The molecule has a single transmembrane sequence followed by a cytoplasmic domain of 123 or 124 amino acids that is highly conserved between the species. On the basis of the high level of glycosylation of leukosialin it has been argued that the extracellular part of the molecule would have an extended structure (Brown *et al.*, 1981).

There is considerable evidence for heterogeneity in leukosialin in terms of apparent M_r (Fukuda and Carlsson, 1986; Remold-O'Donnell *et al.*, 1987), antigenicity (Strandring *et al.*, 1978; Carlsson and Fukuda, 1986; Bettaieb *et al.*, 1988) and glycosylation (Carlsson *et al.*, 1986; Fukuda *et al.*, 1986; Piller *et al.*, 1988). This heterogeneity must be ascribed to post-translational modifications for mouse and human leukosialin because in these species there is only one functional leukosialin gene which lacks introns in the coding sequence (Cyster *et al.*, 1990; Shelley *et al.*, 1990), precluding diversity introduced by alternative splicing of exons.

Antigenic heterogeneity has been seen in human leukosialin in terms of determinants that are specific either for T lymphocytes, or for a larger form on activated lymphocytes and granulocytes (Carlsson and Fukuda, 1986). For some epitopes, antigenicity is reduced if cells are treated with neuraminidase (Sportsman *et al.*, 1985; Axelsson *et al.*, 1988; Stoll *et al.*, 1989). These results might be taken to suggest that leukosialin determinants involve both a protein and a carbohydrate component. The antigenicity of leukosialin is given added interest by the recent finding that anti-leukosialin antibodies are commonly found in patients infected with HIV-1 (Ardman *et al.*, 1990).

In this paper we show that the extracellular part of rat leukosialin has an extended structure and study the relative roles of protein and carbohydrate in the antigenicity of the molecule. We establish epitope positions in the molecule by the complementary techniques of molecular biology and electron microscopy.

Results

Electron microscopy of leukosialin

The molecular structure of the leukosialin molecule was clearly revealed by electron microscopy after low angle rotary shadowing (Figure 1). In the absence of detergent, rosette-like structures were seen, with a variable number of arms of equal length and a central density of size roughly proportional to the number of arms (Figure 1A). The addition of deoxycholate almost completely dissociated these rosettes into individual flexible rods, without discernable polarity or regional specialization (Figure 1B). It is concluded that the rosettes consist of leukosialin molecules that have formed micelles by association of their hydrophobic transmembrane domains, while the structures seen in the presence of deoxycholate are single extended leukosialin molecules.

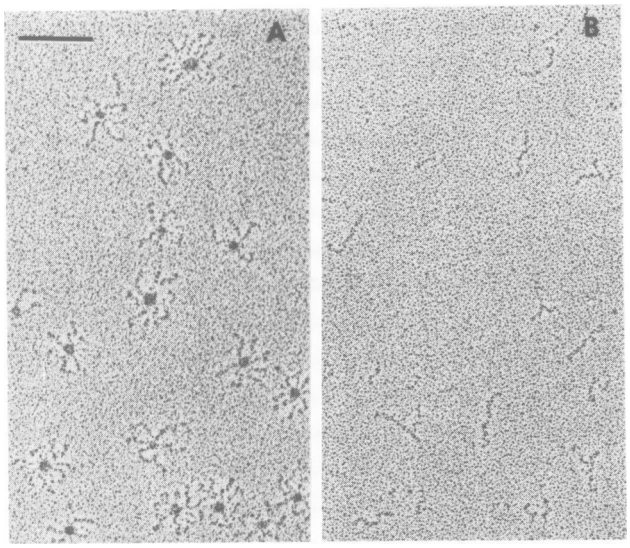

Fig. 1. Electron micrographs of rat thymocyte leukosialin following low angle rotary shadowing. (**A**) Leukosialin micelles in the absence of detergent. (**B**) Leukosialin monomers sprayed from a solution containing .05% sodium deoxycholate. Bar = 100 nm.

The length of the structures as seen in Figure 1 can be measured with reasonable accuracy and a correction of −1 nm for shadow thickness is applied at each free end of the rods (Shotton *et al.*, 1979). In contrast, the widths were too narrow for meaningful measurement. Measurements of length were made for 70 arms of the rosettes illustrated in Figure 1A, using the criteria that the longest straight arms were chosen and measured from the edge of the central density. The mean length ± SD was 41 ± 3.3 nm after the −1 nm correction was applied. Measurements were also made of single molecules shown in Figure 1B with straight forms that were clearly isolated in the micrograph being chosen. The mean length ± SD after corrections of −2 nm was 49.3 ± 7.7 nm from 47 values.

The extra length of single molecules compared with the arms of the rosettes could be due to shadowing of the transmembrane and cytoplasmic segments or to part of the extracellular segment that has collapsed onto the centre of the protein micelle. It seems that the latter possibility will be part of the explanation since a globular cytoplasmic domain of 124 amino acids will be expected to be less than 4 nm in length and if the structure were extended it might not be visualized by this technique. In the case of the CD45 leukocyte molecule the extracellular part was found to overlay the cytoplasmic domain in shadowing studies (Woollett *et al.*, 1985). Thus in subsequent discussion we take a value of 45 nm, which is the mean between the measurements from Figure 1A and 1B, as the length of the extracellular segment of leukosialin.

Antigenic heterogeneity as seen with leukosialin on transfected cells

Full length cDNA for rat leukosialin in either the pHSE3′ or CDM8 eukaryotic expression vector, was transfected into mouse NSO myeloma cells, human Jurkat T lymphoma cells and Madin−Darby canine kidney (MDCK) epithelial cells. Leukosialin on the transfected cells was studied by labelling with nine different monoclonal antibodies, eight of which were of the IgG class. All the MAbs gave strong binding to

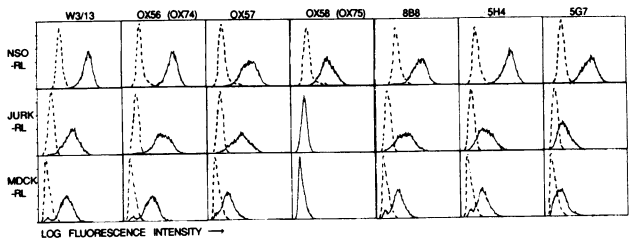

Fig. 2. Binding of MAbs to rat leukosialin expressed on NSO, Jurkat or MDCK cells transfected with rat leukosialin cDNA. Transfected cells (10^6) were incubated with saturating levels of the MAbs followed by a second incubation with fluorescein-labelled $F(ab')_2$ anti-mouse IgG antibody, and bound antibody was measured by flow cytometry. The solid lines show labelling of the transfected cells and dotted lines non-transfected control cells. The horizontal axis shows log fluorescence intensity and the vertical axis log cells per channel. Each horizontal row is for one cell type and each vertical row for one MAb. The profiles for OX-74 and OX-75 were indistinguishable from those for OX-56 and OX-58, respectively, and thus the data for OX-74 and OX-75 are not shown separately.

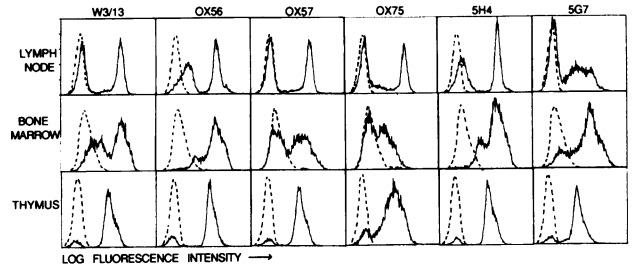

Fig. 3. Binding of anti-rat leukosialin MAbs to rat lymphoid cells analysed by flow cytometry. Cells were isolated from the lymph nodes, bone marrow and thymus of a PVG rat and analysed with anti-rat leukosialin antibodies as described for Figure 2. Profiles for six MAbs are shown. In each case the solid line represents the indicated MAb and the dotted line an isotype matched control. Other MAbs matched one of the patterns shown as follows: OX-58 = OX-75; OX-74 = OX-56; 8B8 = 5H4 and thus the data are not shown separately. Other experiments on PVG and AO rats gave similar profiles.

leukosialin on NSO cells (Figure 2) proving that they recognized the product of one leukosialin gene. In contrast, with the OX-58 and OX-75 MAbs, leukosialin was not labelled at all on Jurkat and MDCK cells, and some of the other MAbs gave weaker binding on these cells in comparison with that of the OX-56 and OX-74 MAbs. It is presumed that these antigenic differences are due to the effects of differential glycosylation.

Labelling of lymphoid cell populations with the anti-rat leukosialin MAbs

All of the MAbs reacted with most thymocytes and T lymphocytes in the lymph node cell population (Figure 3). In contrast the labelling of B lymphocytes in the lymph node population was not distinguishable from that of the negative control, except with the OX-56 and 5H4 MAbs which gave weak binding. Thus it seems that OX-56 and 5H4 detect leukosialin at low levels on B cells. The alternative that they both cross-react with another molecule is unlikely, since these MAbs recognize different epitopes (see below) and it is improbable that different cross-reactions would yield a similar pattern of binding.

With bone marrow cells clear differences in labelling were

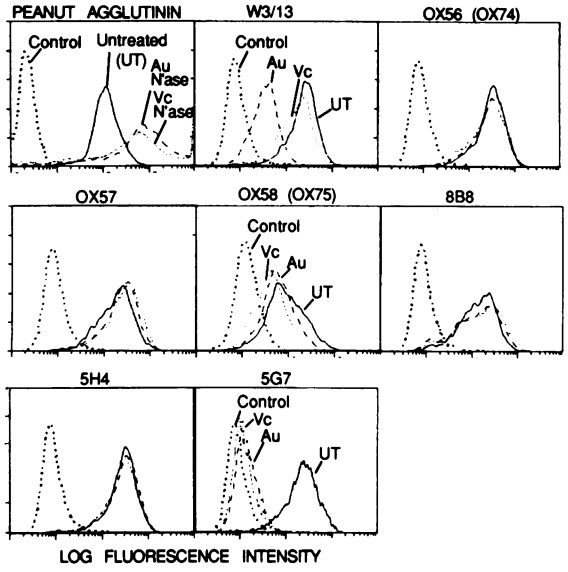

Fig. 4. Effects of neuraminidase treatment on antigenicity of rat leukosialin assayed by antibody binding to cells. Transfected NSO cells expressing rat leukosialin were treated with either *V.cholera* (····) or *A.ureafaciens* (- - -) neuraminidase for 60 min or left untreated (——), or washed in PBS/BSA and analysed directly by flow cytometry as in Figure 2. The first profile shows binding of FITC-conjugated peanut agglutinin before and after the neuraminidase treatments as compared to the background where galactose was present in excess [50 mM, control (···)]. The remaining profiles show the binding of anti-rat leukosialin MAb before and after the neuraminidinase treatment as compared to a negative control antibody OX-21 (···). As in Figure 2, the profiles for OX-74 and OX-75 were indistinguishable from those for OX-56 and OX-58 respectively, and are not shown separately.

seen. The W3/13, OX-57, OX-75 and 5G7 MAbs labelled various subsets of the total cell population while the OX-56 and 5H4 MAbs labelled all the cells. W3/13 labels polymorphs among bone marrow cells but does not bind to the lymphoid cells which are mostly of pre-B lineage (Williams *et al.*, 1977). The OX-56 and 5H4 MAbs must be labelling pre-B cells as well as polymorphs and this correlates with the finding that these MAbs weakly label mature B cells. Thus in the bone marrow population there are clear differences in the recognition of determinants of the leukosialin molecule by different MAbs.

Effect of neuraminidase digestion on the antigenic determinants

Treatment with neuraminidase of NSO cells expressing rat leukosialin was effective in revealing ligands for peanut agglutinin and in producing a significant reduction of binding of four of the anti-leukosialin MAbs (Figure 4). Furthermore, the results obtained differed depending on the type of neuraminidase used. *Arthrobacter ureafaciens* neuraminidase is selective for the removal of α 2-6 linked sialic acid, and this enzyme had more effect on the binding of W3/13 MAb than digestion with *Vibrio cholera* neuraminidase which has a higher activity on α 2-3 linkages (Uchida *et al.*, 1979). In contrast the opposite result was seen for the binding of OX-58 and OX-75 MAbs, whilst digestion with either enzyme resulted in loss of 5G7 binding. Figure 4 also shows that neuraminidase digestion gave a slight enhancement of OX-57 MAb binding.

The effects of neuraminidase treatment were also analysed

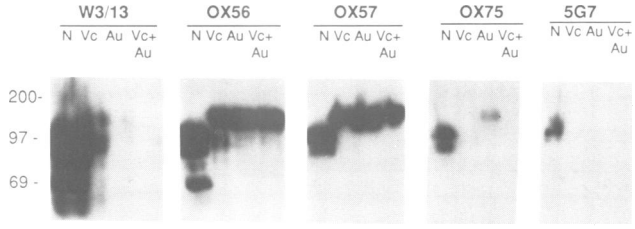

Fig. 5. Effects of neuraminidase treatment on antigenicity of leukosialin as assayed by Western blotting. Transfected NSO cells expressing leukosialin were left untreated (Native, N), treated with *V.cholera* neuraminidase (Vc), *A.ureafaciens* neuraminidase (Au) or a combination of the two (Vc + Au). 10^6 cells were then boiled in 100 μl of reducing sample buffer and 10 μl was run on a 7.5% SDS−PAGE and electroblotted to nitrocellulose. The nitrocellulose membranes were probed with the indicated antibodies and [^{125}I]RAM as the second antibody prior to autoradiography. Numbering on the left indicates the apparent M_r determined from marker proteins (kd).

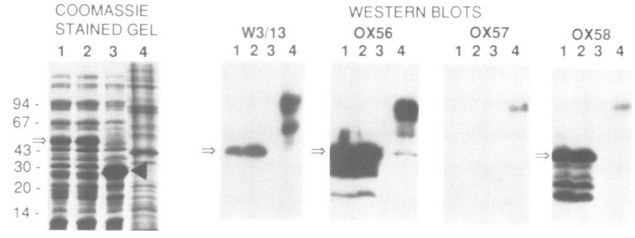

Fig. 6. Reactivity of MAbs with recombinant (non-glycosylated) extracellular domain of rat leukosialin. Coomassie stained gel: Samples of *E.coli* clones expressing the full length extracellular domain as a fusion protein with glutathione-S-transferase (lanes 1 and 2, fusion protein indicated by ⇒) or the glutathione-S-transferase alone (lane 3, GST indicated by arrowhead) were boiled in sample buffer and run on 12% SDS−PAGE. A sample of NSO cells expressing rat leukosialin in glycosylated form was included in lane 4. Western blots: identical gels were Western blotted and probed with the indicated MAb and [^{125}]RAM second antibody prior to autoradiography. Numbering on the left indicates apparent M_r (kd).

by Western blotting after SDS−PAGE (Figure 5), giving results that qualitatively supported the cell binding data. In addition the results in Figure 5 established that both the *A.ureafaciens* and the *V.cholera* neuraminidase had removed substantial amounts of sialic acid from the leukosialin molecule, since the characteristic shift of leukosialin to a higher apparent M_r on SDS−PAGE (Standring *et al.*, 1978; Brown *et al.*, 1981) was seen in both cases.

Binding of MAbs to the rat leukosialin extracellular domain expressed in E.coli

The extracellular domain of rat leukosialin was expressed in *E.coli* as a fusion protein with glutathione-S-transferase (GST) in the pGEX vector system (Smith and Johnson, 1988). The binding of MAbs to the fusion protein was assessed by Western blotting. Of the nine MAbs available, seven gave positive binding reactions. Figure 6 shows the binding obtained with W3/13, OX-56 and OX-58 MAbs and the lack of reaction with the OX-57 antibody. W3/13 MAb bound to a form of the fusion protein having the apparent M_r expected for the full length protein. OX-56 and OX-58 bound both to this form and to lower M_r forms believed to be degradation products. These differences in binding of the MAbs to

Table I. Summary of the data for anti-rat leukosialin MAbs including results for the plate binding assay with fusion protein

MAb	Antibody class	Cell type variation	Neuraminidase sensitivity	Western blot reactivity		Plate binding assay		
				Native leukosialin	Fusion protein	Reactivity scored	Binding data (c.p.m.)	
							GST	GST-fusion
OX21	IgG1						242	154
W3/13	IgG1	+	+	+	+	?+	87	498
OX56	IgG2b	−	−	+	+	+	160	8324
OX57	IgG2b	+	−	+	−	?+	368	929
OX58	IgG2b	++	+	+	+	+	469	9421
OX74	IgG1	−	−	+	+	+	1188	12045
OX75	IgG2b	++	+	+	+	+	803	8369
8B8	IgM	+	−	+	+	+	241	9738
5H4	IgG1	+	−	+	+	+	302	8210
5G7	IgG1	++	+	+	−	?+	222	680

degradation products are consistent with the epitope mapping described below.

The antigenicity of the affinity-purified fusion protein was also tested in a plate binding assay with the results shown in Table I. Binding to the fusion protein in this assay was clearly significant for six of the seven MAbs that showed positive binding after Western blotting. The exception was the W3/13 MAb, for which binding to the fusion protein was low, although above that of the GST control. It was also of interest that the OX-57 and 5G7 MAbs gave measurable binding above the control even though these MAbs were not active in Western blotting. It is perhaps not surprising that the results from the plate assay and from Western blotting were not totally concordant, given that the conformation of the protein might differ in these two assay systems.

Epitope localization by fusion protein expression

To localize epitopes, the plasmid containing the GST−rat leukosialin construct was cut with *Eco*RI to generate linear DNA ending on the 3′ side of the cDNA encoding the extracellular segment. This was further digested with Bal31 exonuclease for various periods to yield fragments with different lengths of 3′ DNA eliminated. The digested material was then cleaved with *Bam*HI to release fragments of the leukosialin cDNA which were recloned into the pGEX vector and expressed in *E.coli* for Western blotting with MAbs. A succession of clones was detected that reacted with different MAbs as the size of the construct increased. For finer mapping, the size of inserts was determined by PCR on bacteria picked from colonies, and further clones were selected with sizes intermediate between those that had already been studied by Western blotting.

Results for the Western blotting of the polypeptides expressed from 13 constructs are shown in Figure 7. It should be noted that the stability of the fusion proteins varied considerably and in a number of cases a fusion protein band was not visible amongst the other *E.coli* proteins after Coomassie blue staining. However, for all constructs with leukosialin fragments of 65 residues or greater, expression could be confirmed by Western blotting with OX-56 and OX-75 MAbs (Figures 7 and 8). To ensure the production of fragments shorter than 65 residues, constructs were made by PCR techniques containing the 23, 39 and 55 NH_2-terminal amino acids of leukosialin. Fusion proteins expressed from these constructs were clearly visible with Coomassie blue staining after SDS−PAGE. Thus the negative

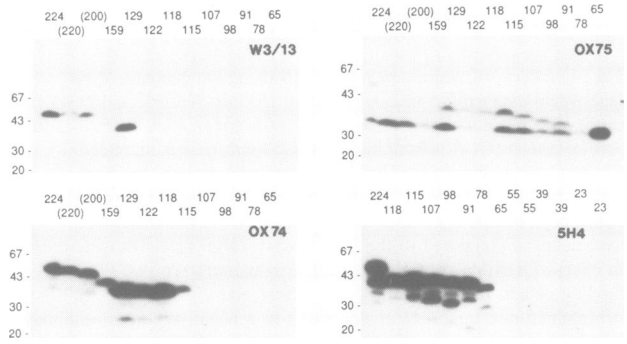

Fig. 7. Epitope mapping of rat leukosialin using deletion clones expressed in *E.coli*. Plasmids containing coding sequences for the extracellular part of leukosialin with deletions at the 3′ end were expressed as fusion proteins in *E.coli*. The cells were then boiled in sample buffer and run on 15% SDS−PAGE for Western blotting. The membranes were probed with the indicated MAb and [^{125}I]RAM as second antibody prior to autoradiography. Numbering above the lanes refers to the leukosialin sequence number at the COOH-terminus of the fusion protein Western blotted in the underlying lane. Values in parentheses are approximate end points, estimated from insert size only, whereas all other end points were determined by sequence analysis. Numbers on the left of the blots indicate apparent M_r (kd). Reactive degradation products of the fusion proteins are evident in many of the lanes in which positive clones had been run. These were not seen in cases where the fusion protein was non-reactive.

antibody binding reactions recorded for these fragments (Figures 7 and 8) were not due to a lack of expression. The epitopes were mapped for all the MAbs that react on Western blotting (Figure 8) and the localization was clear-cut in all cases except that of W3/13, whose epitope was difficult to localize exactly because variable reactivity was seen with clones smaller than residues 1−129. Weak reactivity, not evident in Figure 7, was seen with clones 1-122, 1-120 and 1-118, but no binding at all was seen to clone 1-115 despite repeated blots with this construct. Thus the W3/13 epitope is assigned to the region 116−129. All the epitopes were found to be localized to one or other of two general regions of the extracellular domain, namely residues 55−79 and 107−130 (Figure 8). The data in Figures 7 and 8 firmly establish a physical limit to the extent of epitopes on the COOH-terminal side, but leaves open the possibility that some residues other than those assigned might be necessary on the NH_2-terminal side.

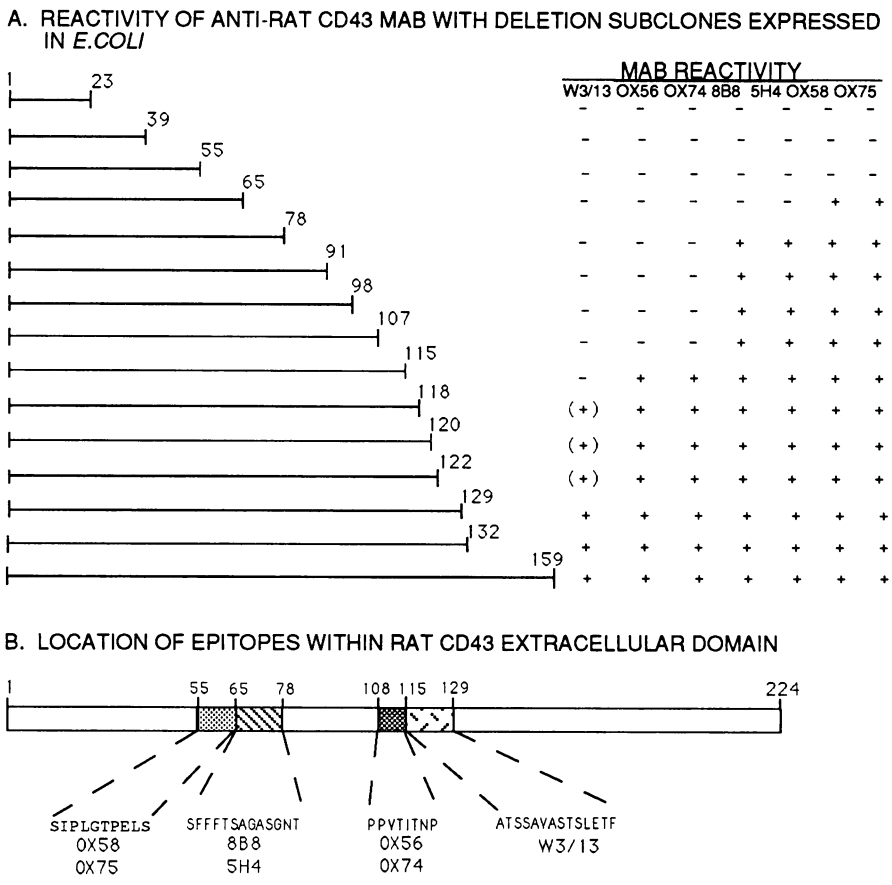

Fig. 8. Summary of the reactivity of anti-leukosialin MAbs with deletion clones of rat leukosialin expressed in *E.coli*. (**A**) The clones were analysed for reactivity by Western blotting with all the antibodies showing clear differences between positive and negative clones with the exception of MAb W3/13, which showed varied reactivity with clones 1-118, 1-120, and 1-122 on repeated blotting. The horizontal lines represent the clone inserts where residue 1 is the NH_2-terminal amino acid of mature leukosialin and the number on the right is the COOH-terminal residue of each deletion clone. A further 4–7 amino acids derived from the vector may be present at the COOH-terminus (see Materials and methods). (**B**) The location of the epitopes in the extracellular domain is shown to scale. The shaded areas correspond to the region between the longest negative and shortest positive clone reactive with the indicated antibody, except with the W3/13 epitope where the shortest 'positive' subclone was taken as 1–129. The epitopes for OX-56 and OX-74 were found to be totally overlapping by this analysis, as were the OX-58 and OX-75 epitopes and the 8E8 and 5H4 epitopes, confirming the similar flow cytometry results obtained for these pairs of antibodies (Figures 2–4).

To confirm the assigment in one case, the epitopes recognized by the OX-56 and OX-74 MAbs were investigated. These epitopes had been mapped by Western blotting of the deletion proteins to the sequence P (108) PVTITNP (Figure 8). An alignment of the rat and mouse leukosialin sequences around this region is shown below, where the dots correspond to sites of *O*-glycosylation in rat leukosialin.

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                 •     •     •     •
Rat    98 F P KS S G VASDPPVT IT NP A T 117
Mouse  95 L P EP S N VASDPPVT AA NP V T 114
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From this alignment and the observation that OX-56 and OX-74 do not react with mouse leukosialin, the residues Ile Thr were candidates for determining the epitope. In transferring the rat epitope to mouse leukosialin, two extra COOH-terminal residues of rat sequence were included (see Materials and methods). These extra residues were thought unlikely to be contact amino acids but might influence the local conformation of the peptide. Thus changes were made in mouse residues 109, 110 and 113 and a fusion protein created with a stop codon after Thr114 (mouse numbering). In a separate experiment, the octapeptide PPVTITNP alone was expressed as a fusion protein with GST. Results from Western blots of these constructs are shown in Figure 9, where it can be seen that OX-74 and OX-56 reacted with the mutated mouse molecule but not with the wild-type form. Furthermore the octapeptide fusion protein gave a clear reaction albeit at a somewhat lower level than might have been expected from the control rat sequence on the same blot. The quantitative aspects of these experiments are difficult to assess because of possible variations including degradation, transfer to the blots, or decrease in antibody affinity due to an incompletely constituted epitope. The clear conclusion, however, is that the OX-56 and OX-74 antibodies can recognize the octapeptide. The complete epitope is likely to be contained within residues 104–115 since the deletion clone finished at rat 115 and residues prior to rat 104 are unlikely to be involved due to the sequence differences between rat and mouse leukosialin in this region.

Localization of epitopes determined by electron microscopy

To visualize epitope positions in glycosylated leukosialin, complexes were made between pure leukosialin and Fab fragments of the W3/13, OX-57 and OX-75 MAbs. According to Figure 8, OX-75 and W3/13 would be expected to bind near the distal end and middle, respectively, of the arms of the rosette form of leukosialin. This result was clearly

seen in the complexes visualized by electron microscopy (Figure 10 A, B) confirming the predicted orientation of the leukosialin molecule in the rosettes. The epitope for the OX-57 MAb could not be mapped with the fusion proteins, because no reaction was seen with the *E.coli* material in Western blotting. However, it seemed possible that this MAb reacted near the NH_2-terminus because it was very potent in causing aggregation of rat thymocytes (data not shown). This possibility was confirmed by low angle shadowing which showed binding of OX-57 at or near the ends of the rosette arms (Figure 10C).

To quantify the differences in position of the W3/13 Fab compared with the OX-75 or OX-57 Fabs, distances were measured between the outer face of the central density of the rosette and the inner side of the Fab. More than 100 measurements were made in each case and the mean values ± SE of mean were: W3/13, 11.6 ± 0.3 nm; OX-57, 21.6 ± 0.4 nm; and OX-75, 21.3 ± 0.5 nm. These values clearly support the finding that W3/13 Fab binds closer to the transmembrane sequence than the other Fabs.

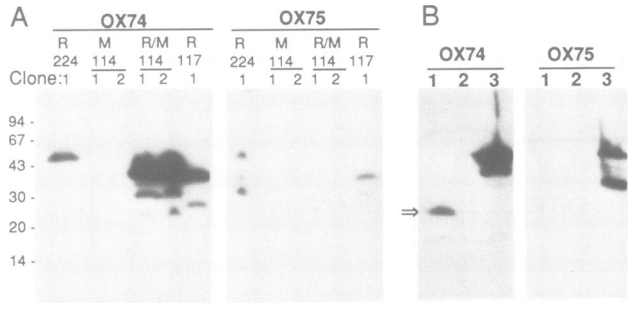

Fig. 9. Reactivity of OX-74 MAb with modified mouse leukosialin and with the octapeptide PPVTITNP. *E.coli* clones expressing fragments of normal or mutated leukosialin, or the octapeptide, as fusion proteins with GST were boiled in sample buffer, run on 15% SDS–PAGE and Western blotted with OX-74 or with OX-75 as a control. In (**A**) the tracks are: R 224 clone 1, rat leukosialin residues 1–224; M 114 clones 1 and 2, two separate clones containing mouse leukosialin residues 1-114; R/M 114 clones 1 and 2, two clones containing mouse leukosialin residues 1-114 with amino acids 109, 110 and 113 mutated to rat sequence; R 117 clone 1, rat leukosialin residues 1-117. In (**B**) the tracks are: 1, octapeptide (rat leukosialin residues 108-115) indicated ⇒; 2, GST alone, and 3, rat leukosialin residues 1-224. Numbering on the left indicates the apparent M_r determined from marker proteins. The OX-56 MAb showed similar reactivity with the modified mouse construct and detectable but weaker reactivity with the octapeptide-fusion protein.

Discussion

In this study we have determined the dimensions of the extracellular part of the leukosialin molecule and have established that the antigenic determinants are largely based on linear protein sequences, with glycosylation having a modulating influence on epitope conformation or accessibility.

The visualization of leukosialin by electron microscopy shows an extended conformation for the glycosylated 224 amino acid extracellular segment of ~45 nm in length. This yields an extension per amino acid residue of ~0.2 nm which is less than the theoretical value of 0.34 nm per residue for a fully extended peptide backbone and greater than the 0.15 nm per residue seen in an α helix. A value of 0.25 nm per amino acid residue has been determined for porcine and ovine submaxillary gland mucins (Shogren *et al.*, 1989; Jentoft, 1990). Maintenance of the extended conformation in the porcine and ovine mucins required the *O*-linked sugars to be present, although the major chain-stiffening effects could be attributed to steric interactions between the peptide-linked GalNac and adjacent amino acids in the peptide core. Residues attached directly to the GalNac had some effect on chain stiffness while more peripheral residues were not involved (Rose *et al.*, 1984; Shogren *et al.*, 1986; Gerken *et al.*, 1989). Similarly, in the case of the cell surface mucin called episialin (epitectin), removal of sialic acid did not affect the extended conformation (Bramwell *et al.*, 1986). These data indicate that changes in the outer regions of the carbohydrate structures attached to the leukosialin backbone, as occurs for

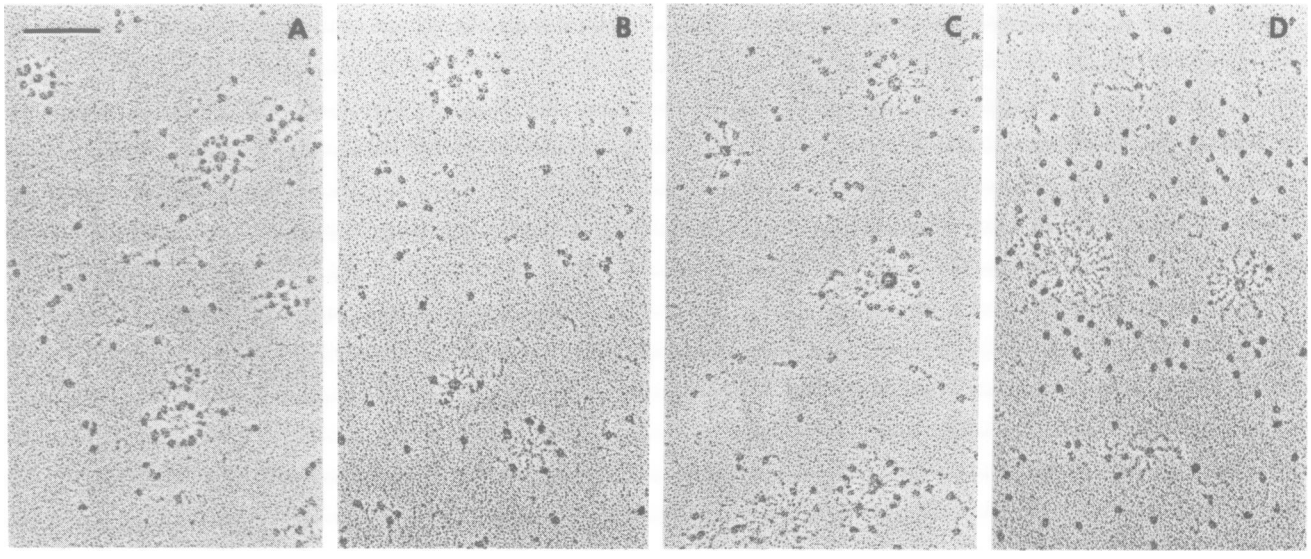

Fig. 10. Electron microscopy of complexes of Fabs and rat leukosialin following low angle rotary shadowing. The complexes were for leukosialin plus Fabs from the anti-leukosialin MAbs: (**A**) W3/13 (**B**) OX-75 (**C**) OX-57. (**D**) shows leukosialin plus unbound Fab from OX-35, a control MAb that is specific for CD4. All complexes were prepared in the absence of detergent. Bar = 100 nm.

example in T cell activation (Piller *et al.*, 1988), should not alter the dimensions of the extracellular domain.

Immunochemical studies indicate that the major glycoproteins of rodent T lineage cells include the leucocyte-common antigen (CD45), leukosialin (CD43) and Thy-1 for thymocytes and CD45, CD43 and MHC class I antigen for T lymphocytes. Other glycoproteins, including the T cell receptor (TCR) and the CD2, CD4, CD5, CD8 and LFA-1 antigens, are more minor constituents on T cells. The topology of the three major glycoproteins of thymocytes can be envisaged as illustrated in Figure 11. Leukosialin and CD45 are likely to extend much further from the cell surface than Thy-1, and the TCR, MHC antigens and CD2 have dimensions only marginally larger than Thy-1. In the case of the CD45 antigen, extra segments shown as A, B, or C in Figure 11 can be found at the NH_2 terminus in mature T and B lymphocytes, due to alternative splicing of exons (Thomas, 1989). These segments have a high content of *O*-linked sugars and are therefore likely to have an extended conformation similar to leukosialin. Thus the B lymphocyte form of CD45 which includes the A, B and C segments, would be expected to have a total extracellular length of ~55 nm with 28 nm derived from the extracellular region (Woollett *et al.*, 1985) and 27 nm from the 140 amino acids of the A, B and C segments. On the basis of the models in Figure 11, leukosialin and CD45 are likely to be the surface molecules that most readily make contact with other cells as lymphocytes move through the tissues.

Leukosialin antigenic determinants are of interest as

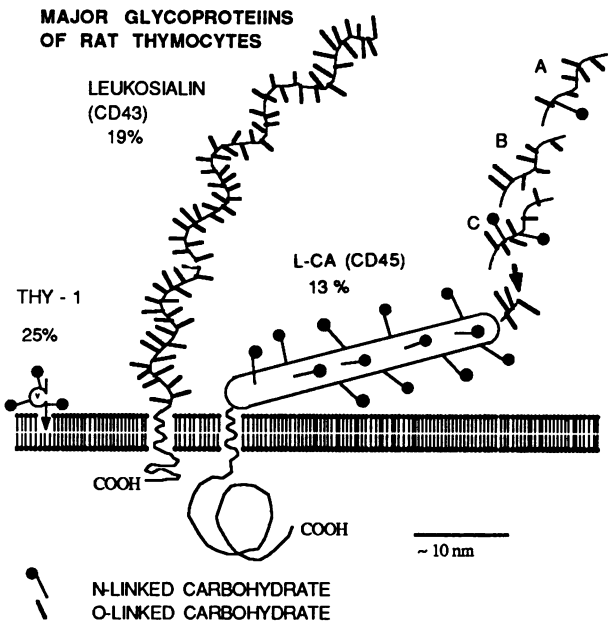

Fig. 11. A model of the major glycoproteins of rat thymocytes drawn roughly to scale. The dimensions for Thy-1 are based on an Ig fold and the Stokes' radius of the molecule (Williams and Barclay, 1986) while those for leukosialin and CD45 are based on electron microscopy from this study and from Woollett *et al.*, 1985. The symbols ↑ and | indicate *N*-linked and *O*-linked carbohydrate respectively. The figures beside each molecule represent the percentage of the rat thymocyte cell surface that is calculated to be covered by each of these molecules. For these calculations the thymocyte was taken to have a smooth surface and a volume of 125 μm^3, Thy-1 was taken as a sphere of radius 3 nm, and leukosialin and CD45 as rods of 45 × 5 and 28 × 8 nm respectively. The site numbers were taken to be 10^6, 10^5 and 7×10^4 respectively (see Williams and Barclay, 1986 for a background to these calculations).

examples of linear epitopes, and an understanding of their structural basis provides an indication as to whether the protein part of the extracellular domain is obscured by carbohydrate or free for interaction with other molecules. The surprising finding was that epitopes for seven of the nine MAbs studied here were expressed in the unglycosylated protein chain made in *E.coli*. This included the W3/13, OX-58 and OX-75 MAbs for which there is strong evidence that the epitopes recognized are affected by glycosylation. For all these MAbs, neuraminidase treatment of leukosialin affects binding, and for OX-58 and OX-75 the expression of epitopes on transfected leukosialin is affected by the host cell type. It could be argued that epitopes for all the other MAbs except OX-56 and OX-74 are also affected by glycosylation, on the basis that they show different patterns of labelling on normal lymphoid cells. However, there is the caveat that for rat leukosialin there could be more than one functional gene (Cyster *et al.*, 1990) and if so different cell types might use different genes.

The position of epitopes was mapped for seven of the MAbs by expressing fusion proteins of different lengths, and the assignments were confirmed in two cases by electron microscopy on Fab–leukosialin complexes. The overall conclusion was that each epitope was determined by a single stretch of protein sequence. Laver and colleagues have recently queried the existence of linear epitopes in native protein structures (Laver *et al.*, 1990) but the epitopes of leukosialin would appear to be unambiguous examples in this category.

We conclude that the specificity of leukosialin epitopes is largely determined by linear protein sequence, but that their conformation or accessibility can be modified by glycosylation. The M/N antigenic determinants of glycophorin A provide the classical example in which antigenicity appears to be affected by both protein sequence and carbohydrate, but in this case the antigenicity of the unglycosylated form has not, to our knowledge, been characterized (Lisowska and Wasniowska, 1978; Sadler *et al.*, 1979; Prohaska *et al.*, Lisowska *et al.*, 1987). In another study, a MAb specific for human fibronectin was shown to react with the peptide VTHPGY, but only when the Thr residue had an *O*-linked sugar attached (Matsuura *et al.*, 1988). It was further demonstrated that the minimal carbohydrate requirement for antigenicity was the presence of GalNAc. Results more similar to the leukosialin data have recently been reported for the cell surface mucin, episialin. In this case MAbs were found that reacted with native, but not asialo episialin, yet these MAbs reacted with β-galactosidase–episialin fusion proteins isolated from λgt11 expression cloning systems (Siddiqui *et al.*, 1988; Ligtenberg *et al.*, 1990; Gendler *et al.*, 1990). In addition an immunodominant region of the episialin molecule has been studied by synthesizing peptides corresponding to the mucin repeat segment (Price *et al.*, 1990). A number of antibodies whose binding had been suggested to involve carbohydrate (Burchell *et al.*, 1983; Sekine *et al.*, 1985) were shown to bind the unglycosylated peptides. It may be the general case that carbohydrate can modify linear protein epitopes rather than being part of the contact sites for antibody binding.

The finding that the leukosialin antigenic determinants are linear protein epitopes is relevant to thinking about the functions of such glycoproteins. There are no known molecules that recognize the extracellular parts of leukosialin but clearly this molecule could be a recognition structure for cell interactions based on protein as well as carbohydrate

epitopes. Furthermore the specificity of the protein determinants could be modified depending on the state of glycosylation in different cell types.

Materials and methods

Antibodies and rat leukosialin

The following mouse and anti-rat leukosialin MAbs were used in the form of undiluted tissue culture supernatant, or ascites fluid diluted 1/100 and their isotypes are given in Table I: W3/13 (Williams *et al.*, 1977), immunogen, rat thymocyte membrane; MRC OX-56 (Killeen *et al.*, 1987), immunogen, activated rat T lymphocytes; MRC OX-57, OX-58, OX-74 and OX-75 (unpublished MAbs prepared by W.R.A.Brown and M.Puklavec, MRC CIRU) immunogen, rat thymocyte leukosialin and thymocytes; 8B8, 5H4 and 5G7 (unpublished MAbs prepared by S.Fossum, Anatomical Institute, University of Oslo) immunogen, rat lymphokine activated killer cells. Control MAbs used were MRC OX-21 (IgG_1), mouse anti-human C3b inactivator and MT-463 (IgG_2b), mouse anti-human CD4. Fabs were prepared from MAbs as in Davis *et al.* (1990). [^{125}I]RAM was affinity purified rabbit anti-mouse IgG antibody degraded by pepsin to $F(ab')_2$ and labelled with ^{125}I (Williams *et al.*, 1977). Rat leukosialin was purified from thymocytes as in Killeen *et al.* (1987).

Rotary shadowing and electron microscopy

Purified leukosialin, at 100 μg/ml in 0.1 M NH_4HCO_3 with or without 0.5% sodium deoxycholate, was diluted 10-fold in 77% (v/v) glycerol; 0.1 M NH_4HCO_3 prior to rotary shadowing. Leukosialin−Fab complexes were formed by incubating equal weights of the two proteins overnight at 4°C and then treating as above in the absence of deoxycholate. 50 μl of the samples in glycerol were sprayed without delay as a mist onto the surfaces of freshly cleaved mica sheets, using a laboratory aerosol spray canister, and dried at room temperature by evacuation to better than 2×10^{-5} mbar for 10 min on the uncooled specimen stage of a Balzers BAF 301 freeze-fracture apparatus. They were then rotary shadowed with platinum−carbon at an incident angle of 6° while rotating at 2 Hz as previously described (Shotton *et al.*, 1979; Woollett *et al.*, 1985). Replicas were floated onto distilled water, picked up on bare 360 hexagonal mesh copper grids and photographed on Ilford EM film using a Philips EM 400T electron microscope operated conventionally at 80 kv.

Cells, neuraminidase treatment and flow cytometry

Lymphoid cells from rat lymph nodes, thymus and bone marrow were prepared by conventional procedures (Williams *et al.*, 1977) and washed in phosphate-buffered saline (PBS) plus 0.25% bovine serum albumin (BSA). Jurkat E6-1 and NSO cells were grown in RPMI 1640 medium and MDCK cells in Dulbecco's modified Eagle's medium, both supplemented with 10% heat-inactivated fetal calf serum, L-glutamine, sodium pyruvate and antibiotics. MDCK cells were freed from tissue culture flasks by treatment with trypsin (0.25%) in PBS for 15 min. For treatment with neuraminidase, cells were washed and resuspended in PBS titrated to pH 6 and containing 2 mM $CaCl_2$ at 10^7 cells/ml and incubated with or without 0.05 U of *V.cholera* or *A.ureafaciens* neuraminidase (Calbiochem Corporation, La Jolla, CA) at 37°C for 1 h. Flow cytometry was with standard techniques on a Becton Dickinson FACScan flow cytometer (He *et al.*, 1988). Cells were gated on the scatter profiles to exclude dead cells and erythrocytes.

Preparation of a full length rat leukosialin cDNA clone

The cDNA for rat leukosialin lacked the leader sequence (Killeen *et al.*, 1987). Alignment of mouse and human leukosialin nucleotide sequences in a region 5′ to the initiation ATG codon revealed a patch with 16/21 identities. Thus an oligonucleotide was designed with a *Sal*I site plus 17 nucleotides on the 5′ side of the ATG to give the sequence cgtcgacctgtccctctggagatgg. On the 3′ side an oligonucleotide was made including rat sequence from a region immediately 3′ to the unique *Sty*I site in the coding region (agaagacaa-ttctggagttccc). These oligonucleotides were used in a PCR with rat genomic DNA as template to yield a product of the expected size that reacted with an oligonucleotide derived from known rat sequence 5′ of the *Sty*I site. The fragment was gel purified and treated with *Sal*I and *Sty*I. This fragment was used to replace the incomplete region in a derivative of the original clone for rat leukosialin [pLSGP-2 (Killeen *et al.*, 1987)] to yield a full length clone in the pTZ-19R vector. The full length cDNA was released with *Hin*dIII, blunt ended and inserted into the pHSE3′ vector (Ferrick *et al.*, 1989) cut with *Sal*I and *Bam*HI and blunt-ended. This construct was called pHSE3′-RL. The same cDNA was inserted into the CDM8 vector (Seed, 1987) which had been cut with *Xho*I and blunt-ended. This construct was called CDM8-RL.

The nucleotide and protein sequences of the rat leader peptide used in the constructs were respectively atggccttgcatcttctcctcctctttgggggcttctgggcccaggt-ggtgagccaa, and MALHLLLLFGGFWAQVVSQ.

Transfection of cell lines

Jurkat and NSO cells were transfected by electroporation (He *et al.*, 1988) and MDCK cells by the calcium phosphate technique (Sambrook *et al.*, 1989). Jurkat cells and NSO cells were co-transfected with pHSE3′-RL linearized with *Xho*I and uncut pKG5 (which carried the *neo* gene, He *et al.*, 1988) at a ratio of 5:1 (30−60 μg total DNA). MDCK cells were transfected with linearized CDM8-RL and pKG5 at a similar ratio (30 μg DNA). Cells were selected with 0.8 mg/ml G418, and in the case of the NSO cells, thymocyte feeders were added to rescue small numbers of drug resistant cells. With NSO cells 80% were initially leukosialin positive. This percentage, and the level of leukosialin, increased spontaneously during 5 weeks further culture. With Jurkat and MDCK cells only 20−40% were leukosialin positive at 4 weeks, and the positive cells were thus enriched to >80% by sorting on a Becton Dickinson FACS II after labelling with the W3/13 MAb.

Polymerase chain reaction

PCR was as in Chang *et al.* (1989). Reactions were carried out in a Perkin Elmer Cetus DNA thermal cycler and conditions were: denaturation (1 min, 94°C), annealing (2 min, 60°C) and elongation (1 min, 72°C), for a total of 30 cycles. Colony PCR involved the same conditions except the template was DNA spontaneously released from an *E.coli* colony, containing the plasmid of interest, that was transferred with a toothpick to a tube containing PCR mix (20 μl for insert size analysis or 100 μl for template preparation).

Isolation of DNA encoding extracellular segments

DNA encoding the extracellular segment of rat leukosialin, or fragments of rat and mouse leukosialin starting from the NH_2-terminus, were isolated by PCR. The oligonucleotides were designed to yield a fusion protein in-frame with GST in the pGEX-2T vector and to have 6 and 3 nucleotides overhang on the 5′ and 3′ ends and restriction enzyme sites plus 17−20 nucleotides of complementary sequence. The restriction sites were 5′ *Bam*HI, 3′ *Eco*RI. The oligonucleotide sequences were as follows with the beginning of the complementary sequence preceded by a number which is the nucleotide position in a numbering system starting with the first nucleotide of the initiation codon of rat leukosialin cDNA. Rat extracellular segment, 5′ end, tagtagg-atcc(58)gaaaatctgccgaatacg; 3′ side, ctgaattc(729)agccactcgatcctgg. 3′ side oligonucleotides for generation of rat leukosialin fragments: ctagaattc(131)-attcgaggtacttggg; ctagaattc(179)agtcctctgtcactggcac; ctagaatcc(227)aggaagc-tggggcagtg. Mouse leukosialin extracellular fragment 5′ end: tagtaggg-atcc(58)ctgcagaggacgacgatg 3′ side, ctagaattca(399)tgtcacaggattggctgcagtga-cagga, and 3′ side with mutations to rat codons at three positions, ctagaatt-ca(399)tgtcgcaggattggttatagtgacagga. Single stranded DNA was used as template, and PCR products were phenol extracted, ethanol precipitated, treated with *Bam*HI and *Eco*RI and ligated into the pGEX-2T expression vector (Smith and Johnson, 1988) which had been treated with the same enzymes. The plasmid with the whole rat extracellular domain was termed pGEX-RLex. The ligated DNA was used to transform either *E.coli* MC1061 or TG1 as described by Sambrook *et al.* (1989). For expression of the octapeptide PPVTITNP two complementary oligonucleotides were made which included 5′ and 3′ overhangs that would generate open *Bam*HI and *Eco*RI sites following annealing: sense, gatcccctcctgtcactataactaatccttg; anti-sense; aattcaaggattagtta-tagtgacaggaggg. The oligonucleotides were treated with T4 polynucleotide kinase as described by Mallet *et al.* (1990). They were then ligated into the pGEX-2T vector which had been treated with *Bam*HI and *Eco*RI, annealing occurring during the 4 h ligation reaction at 16°C. Oligonucleotides specific for pGEX-2T 5′ and 3′ of the multiple cloning sites were used for analytical PCR and sequencing, 5′ side: gcaagccacgtttggtg, 3′ side: ttcaccgtcatcaccga.

Expression in E.coli

This was principally as described by Smith and Johnson (1988). In brief, overnight cultures were diluted 1/10 (to 5 ml) in fresh medium and grown for 1 h before addition of IPTG to 1 mM and a further 3 h growth. The cells were then pelletted, resuspended in 300 μl sample buffer and boiled for 5 min. 3−5 μl were used in SDS−PAGE Western blotting analysis. Affinity purification of the full length extracellular domain fusion protein was as described by Smith and Johnson (1988).

Generation of clones by Bal31 deletion

The pGEX-RLex plasmid was linearized with *Eco*RI. 30 μg was treated with 12 μl of *Bal*31 in a total of 500 μl (600 mM NaCl, 12.5 mM $CaCl_2$, 20 mM Tris pH 8, 1 mM EDTA) at 30°C with 100 μl aliquots taken at 1, 2, 3.5, 5, 7.5 and 10 min and mixed with 10 μl 0.5 M EDTA to stop the reaction.

The samples were phenol extracted, ethanol precipitated, and resuspended in 200 μl of 10 mM Tris pH 8, 100 mM NaCl, 7 mM $MgCl_2$, 7 mM 2-mercaptoethanol buffer with 0.1 mM dNTPS and 10 U of Klenow. After 30 min at 37°C the DNA was re-extracted and pecipitated before being taken up in 100 μl of the above buffer and treated with *Bam*HI at 37°C for 2 h. 7 μl samples were analysed for size by agarose gel electrophoresis, and the fragments were purified by gel fractionation. The purified fragments were ligated into the pGEX-2T vector that had been cut with *Bam*HI and *Sma*I and treated with phosphatase. Resultant clones were grown and analysed by Western blotting and PCR. The pGEX-2T vector has stop codons present in all three reading frames 4, 6, or 7 codons past the *Sma*I restriction enzyme site. Thus, depending on the digestion end point, expressed fusion proteins could have the residues XGIHRD, GNSS, or XEFIVTD at the COOH-terminus where X is variable. To identify constructs with inserts of particular size, colonies corresponding to the appropriate digestion time point were picked and analysed by PCR using oligonuceotides specific for vector sequences 5′ and 3′ of the cloning site. The insert size was determined by agarose gel electrophoresis. The end points of the clones of interest were determined by sequencing the PCR products (Winship, 1989).

Western blotting

SDS–PAGE was performed using a mini-gel apparatus (Bio-Rad) and protein was transferred to nitrocellulose (Schleicher & Schuell, Dassell FRG) which was placed in 5% milk powder in PBS for 45 min at 20°C. Then 1/5 diluted tissue culture supernatant or 1/500 diluted ascites fluid containing the appropriate MAb was added for 2 h at 4°C with gentle rocking. The membranes were washed twice for 20 min in PBS/0.05% Tween 20 before addition of [^{125}I]RAM (10^6 c.p.m./ml) in PBS/0.05% Tween 20/1% BSA. After 1 h at 4°C the membranes were washed twice for 10 min each with PBS/0.05% Tween 20, and then exposed to X-OMAT S film (Kodak, France) at −70°C, usually for 16 h.

Plate binding assay

Purified GST-fusion protein or GST alone each at 50 μg/ml were used to coat flexible assay plates (Falcon 3911, Microtest III) and used in assays with the first binding step involving incubation with 25 μl MAbs for 40 min at 4°C and the second step a similar incubation with [^{125}I]RAM at 1.5×10^5 c.p.m./well.

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