Characterization of the five novel Ly-6 superfamily members encoded in the MHC, and detection of cells expressing their potential ligands

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Abstract

Lymphocyte Antigen 6 (Ly-6) superfamily members are cysteine-rich, generally GPI-anchored cell surface proteins, which have definite or putative immune related roles. There are 27 members of this family described so far in the human genome and 37 in the mouse. Five of them are clustered in the class III region of the human and mouse MHCs. Following computational analyses, we functionally characterized the encoded proteins by creating epitope-tagged fusion constructs to determine molecular weight, complex formation, subcellular localization, post-translational modifications and ligand binding. We found that all human and mouse proteins were glycosylated, and most could form part of larger complexes. Human and mouse Ly6G6c and Ly6G6d, and mouse Ly6g6e were found to be GPI-anchored cell surface proteins, highly expressed at the leading edges of cells, on filopodia, which are normally involved in cell adhesion and migration. However, analysis of Ly6G5c and Ly6G5b indicated that they are potentially secreted proteins. Our results indicate that there are two subclusters of related Ly-6 proteins in this region of the MHC, with Ly6G6c, Ly6G6d, and Ly6G6e forming one and Ly6G5c and Ly6G5b forming another. In addition, by FACS analysis we have found that the potential ligands for human LY6G6C, LY6G6D, and LY6G5C are expressed on K562 cells, an undifferentiated megakaryocyte cell line, indicating a potential role in hematopoietic cell differentiation. This characterization of the five MHC class III region Ly-6 family members is of great relevance, as they represent 18% of the human Ly-6 protein family and 50% of the secreted ones.

Keywords: Ly-6; MHC; class III; cell differentiation; filopodia; GPI

The Ly-6 proteins are normally glycosyl phosphatidylinositol (GPI)-anchored cell surface, cysteine-rich molecules, such as CD59 (Davies et al. 1989), sperm acrosomal protein (SP-10) (Palfree 1996), and the urokinase plasminogen activator (uPA) receptor (uPAR) (Wilhelm et al. 1999; Stroncek et al. 2004 and references therein). In addition, secreted members of the superfamily lacking the GPI anchor, such as the snake neurotoxins (Fleming et al. 1993) and human SLURP-1 (secreted Ly-6/uPAR related protein 1) (Adermann et al. 1999) and SLURP-2 (Tsuji et al. 2003), as well as secreted rat urinary proteins (Southan et al. 2002) have also been described. The Ly-6 protein domain is ~80 amino acids long and is characterized by a conserved pattern of eight to 10 cysteine residues that have a defined disulfide-bonding pattern. Ly-6 proteins are usually single domain proteins, although a few family members contain more than one Ly-6 domain, such as rat

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RoBo-1 (Noel et al. 1998) and human CD177 (Stroncek et al. 2004 and references therein), which have two Ly-6 domains, and uPAR, which contains three. As yet, the Ly-6 domain has not been found to combine with other functional domains. It has been estimated that only ~2% of cell surface molecules expressed on leukocytes contain a Ly-6 domain, making it a relatively rare domain. Some Ly-6 proteins are expressed on a wide range of tissues, while others have a more restricted pattern of expression. Most hematopoietic cells express one or more members of the Ly-6 superfamily (for review, see Bamezai 2004).

Little is known about the function of Ly-6 proteins, except for CD59 and uPAR. CD59 is an inhibitor of the complement cascade, inhibiting the formation of the membrane attack complex (Davies et al. 1989), and uPAR plays an important role in the proteolysis of extracellular matrix proteins (Tarui et al. 2001 and references therein). Additionally, the Lynx-1 protein has been demonstrated (in vitro) to be a modulator of nicotinic acetylcholine receptors (Miwa et al. 1999), similar to the Ly-6 family snake venom neurotoxins (Tsetlin 1999), while mouse GPI-HBPI has been shown to mediate selective lipid uptake from HDL particles (Ioka et al. 2003), and human/mouse SAMP-14 has been shown to have a potential role in sperm–egg interactions (Shetty et al. 2003). SLURP-1, Lynx-1, and the recently described Lynx-2 (Dessaud et al. 2006) are now grouped as a Ly-6/neurotoxin superfamily. A possible role for some mouse Ly-6 family members in T cell activation, differentiation, and maturation has been suggested (Gumley et al. 1995). In addition, it has been shown that mouse Ly-6C can regulate endothelial adhesion and homing of T cells by activating integrin dependent pathways (Hanninen et al. 1997) and that mouse Ly-6A has a role in mediating cell–cell adhesion (Bamezai and Rock 1995). Although the murine Ly-6 antigens were originally identified as hematopoietic markers, expression of the murine ThB gene was shown to be much higher on keratinocytes than on lymphocytes (Brakenhoff et al. 1995). Ly-6K, Ly-6A/E, Ly-6C, Ly-6F, and RIG-E have also been found to be expressed on keratinocytes (de Nooij-van Dalen et al. 2003). Recently, several studies have revealed the involvement of some Ly-6 family members in different diseases. The SLURP-1 gene has been implicated in a skin disorder, Mal de Meleda (Fischer et al. 2001), CD177 with neutrophil proliferation and polycythemia vera (Stroncek et al. 2004 and references therein); SLURP-2 has been found to be up-regulated in psoriasis vulgaris (Tsuji et al. 2003); and Ly-6K is overexpressed on head-and-neck squamous cell carcinoma (de Nooij-van Dalen et al. 2003).

A Ly-6 gene cluster is located in the class III region of the major histocompatibility complex (MHC) (The MHC Sequencing Consortium 1999; Mallya et al. 2002; Xie et al. 2003). The human MHC is located at chromosome 6p21.3, and it consists of three regions: class I and class II, and the central class III region (Horton et al. 2004). The human MHC has been linked to susceptibility to many diseases and often these associations cannot be fully explained by variation in the class I and II genes. Therefore, the study of novel potential cell surface receptors in the class III region may provide insights into the understanding of these diseases. The (*HUMAN/mouse*) *LY6G6C/Ly6g6c*, *LY6G6D/Ly6g6d*, *LY6G6E/Ly6g6e*, *LY6G5C/Ly6g5c*, and *LY6G5B/Ly6g5b* genes are members of the Ly-6 superfamily of proteins, based on translations of the confirmed gene transcripts (Mallya et al. 2002). The transcripts generated by these MHC-linked Ly6 genes have been previously described (Mallya et al. 2002) and showed a complex pattern of expression in human and mouse cell lines and tissues. This Ly-6 cluster is located in an 80-kb segment of DNA between the TNF and HSP70 genes, in a region where several disease susceptibilities have been mapped, including type 1 diabetes (Herr et al. 2000), rheumatoid arthritis (Zanelli et al. 2001; Newton et al. 2004), and IgA deficiency (Schroeder et al. 1998). Here we present a detailed analysis of the human and mouse MHC class III region encoded Ly-6 proteins.

Results

Comparative protein analysis

The protein sequences of each of the MHC class III region Ly-6 proteins were compared to the NCBI nr database to try to elucidate any potential function. Human LY6G6C seemed to be the most typical Ly-6 family member showing homology with many different Ly-6 family members over the entire protein sequence. The other human MHC class III region Ly-6 proteins showed only limited homology with Ly-6 proteins (data not shown).

BLAST pairwise comparisons of the human, mouse, and predicted pig and rat Ly-6 family members in the MHC class III region were performed (Table 1). Generally, comparisons using either the whole protein sequence or just the Ly-6 and C-terminal domains (data not shown) gave similar values. Between orthologs there is a high percentage of similarity and identity, with Ly6G6c (see nomenclature note in Materials and Methods) being the most highly conserved (83%–96% identity and 88%–97% similarity) (Table 1). However, no significant similarity between different members of the family was found.

A multiple alignment of the MHC class III region Ly-6 proteins in different species (Fig. 1) highlights conserved residues, which are primarily the eight to 10 cysteine residues, a histidine or a tyrosine residue after the first totally conserved cysteine, and the C terminal section of the Ly-6 domain (Cys-Cys-X-X-Asp-X-Cys-Asn). These cysteine residues are involved in forming the structure of the protein, allowing more opportunity for variation of the

Table 1. *Comparison of the human (H), pig (P), rat (R), and mouse (M) MHC class III region Ly-6 proteins*

ID / SIM	Human					Pig					Rat					Mouse				
	G6C	G6D	G6E	G5C	G5B	G6c	G6d	G6e	G5c	G5b	G6c	G6d	G6e	G5c	G5b	G6c	G6d	G6e	G5c	G5b
HG6C					28	84		26	28	27	83				28	84				28
HG6D							66	25				58	24				61			
HG6E							29	70				26	58					66		
HG5C					26				67	26				65	24				69	24
HG5B	39			42		29			27	74	27			25	63	27			26	63
PG6c	88				39				29	24	85			24	27	86			24	27
PG6d		72	35					28				65	24				65	22		
PG6e	38	37	77				36					23	70				25	71		
PG5c	44			76	43	42				28	28			58	29	28			59	30
PG5b	40			45	83	41			45		26			28	64	27			27	65
RG6c	89				38	91			41	40			26		26	96				27
RG6d		65	37				74	36					27				93	25		
RG6e		37	68				33	79			42	39					25	84		
RG5c				73	41	40			70	42					25				82	27
RG5b	36			35	75	39			40	74	40			37		26			25	81
MG6c	89				38	92			41	41	97				39					27
MG6d		68					75	35				93	34					23		
MG6e			71				33	79				38	89				36			
MG5c				73	41	41			70	44				88	36					27
MG5b	35			37	75	38			42	75	38			41	87	38			41	

Percentage identity (ID) (numbers above shaded area) and similarity (SIM) (numbers below shaded area) are shown where significant over >80 amino acids.

other residues, which could potentially contribute to the variety of functions. It is interesting to note that while human LY6G6D has only eight conserved cysteine residues and human LY6G5C has nine, their mouse, pig, and rat orthologs all have 10 cysteine residues.

Each human and mouse MHC class III region Ly-6 protein sequence was analyzed with different bioinformatics programs to predict domains, secondary structure, signal sequence, and transmembrane sequences, as well as possible sites for post-translational modifications (PTMs) (Table 2). The proteins range from 125/126 (human/mouse) amino acids (Ly6G6c) to 201/194 amino acids (Ly6G5b) with high conservation in amino acid length between both species. Human and mouse showed similar but not identical PTMs. LY6G6C/Ly6g6c had the clearest GPI anchor sequence prediction. The LY6G5C/Ly6g5c proteins had some similarity to the C-type lectin domain, a domain responsible for binding to carbohydrates. Interestingly, human LY6G5B showed a very weak potential GPI anchor sequence, and the mouse Ly6g5b protein was not predicted to be GPI anchored. A less detailed analysis was performed in pig and rat, but both species had 10 conserved cysteine residues in all proteins, and all had a predicted signal peptide (data not shown). In rat, Ly6G6c, Ly6G6d, and Ly6G6e were predicted to be GPI anchored (as in human and mouse), whereas in pig only Ly6G6c was clearly predicted, and Ly6G6e only potentially (data not shown).

Experimental protein analysis

The human and mouse MHC class III region Ly-6 genes were studied at the protein level by expressing T7-epitope-tagged constructs in Cos7 cells. Western blot analyses showed that both human and mouse Ly6G6c and Ly6G6d were highly expressed, especially Ly6G6d, but Ly6G5c and Ly6G5b were expressed at relatively lower levels (Fig. 2). LY6G6E is described as a pseudogene in human (Mallya et al. 2002) and for this reason only the two mouse Ly6g6e (Ly6g6e4 and Ly6g6e5) proteins were analyzed. Interestingly, Ly6g6e4 could not be detected by Western blotting (data not shown), while the Ly6g6e5 protein was quite well expressed (Fig. 2). Human and mouse Ly6G5c and Ly6G5b were predicted to be secreted proteins. However, it was not possible to observe expression of these proteins in the supernatant, either when loaded directly on a gel, TCA-precipitated, or immunoprecipitated with the T7.Tag antibody (data not shown).

To examine whether these Ly-6 proteins were monomeric or formed part of larger complexes, they were analyzed in reducing and nonreducing conditions (Fig. 2). Human and mouse Ly6G6c showed bands of 18–26 kDa

Figure 1. Alignment of the human MHC class III region Ly-6 proteins and their orthologs. Human (hum), mouse (mou), pig (pig), and rat (rat) MHC class III Ly-6 sequences are indicated. Human LY6G6E is represented by the 399-bp form, and mouse ly6g6e by the 405-bp form. The connectivity between the conserved cysteines are shown highlighted in gray in the consensus motif.

and 18–33 kDa, respectively, under reducing conditions, but under nonreducing conditions both showed additional bands at ~36–49 kDa (Fig. 2), faint in human but very clear in mouse. Human LY6G6D showed bands of 10–19 kDa and ~24 kDa, and mouse Ly6g6d showed bands of 11–30 kDa in reducing conditions. In the absence of β-mercaptoethanol, both human and mouse Ly6G6d showed an increase in molecular weight, although some protein remained monomeric. Human LY6G6D increased in size to 20–37 kDa, and mouse Ly6g6d to 22–40 kDa (Fig. 2), indicating that the proteins could form homodimers. However, mouse Ly6g6e5 showed bands of 15–20 kDa in both reducing and nonreducing conditions (Fig. 2), consistent with the fact that there is an even number of cysteine residues in the protein and it is thus expected to remain monomeric. However, under nonreducing conditions there are also faint bands at 25–36 kDa, which could indicate the formation of either homo- or heterodimers. Human LY6G5C showed one band of 15 kDa, while mouse Ly6g5c showed two bands of 10 kDa and 13 kDa. In the absence of β-mercaptoethanol, both human and mouse Ly6G5c showed an increase in molecular weight, with LY6G5C showing two bands of 32 kDa and 36 kDa while Ly6g5c showed two bands of ~22 kDa, as well as three bands of ~30–36 kDa (Fig. 2), indicating that these proteins form oligomers. Human LY6G5B showed two bands of ~24kDa, while mouse Ly6g5b showed two bands of ~27 kDa. In the absence of β-mercaptoethanol, human and mouse Ly6G5b showed different expression patterns, as human LY6G5B showed an increase in molecular weight to ~37 kDa, while mouse Ly6g5b remained monomeric.

Post-translational modifications and subcellular localization

It is useful to know the glycosylation patterns of proteins, as sugar residues can be important in protein function (Moller et al. 1993). CD59 is both *N*- and *O*-glycosylated as well as GPI-anchored, and the glycosylation of the protein is thought to contribute to protein structure and stability of the active site (Rudd et al. 1997). Furthermore, the glycosylation status of the Ly-6 molecule uPAR is known to influence its ligand binding ability (Moller et al. 1993). The MHC Ly-6 proteins were predicted to contain glycosylation sites (Table 2) and had higher than expected molecular weights and/or showed a range of

Table 2. *Summary of the predicted post-translational modifications of the human MHC class III region Ly-6 proteins*

	Gene				
Type of modification	Ly6G6c	Ly6G6d	Ly6G6e*	Ly5G5cB	Ly6G5b
No. of cysteines	**10**	**8**, 10	**10**	**9**, 10	**10**
Length (bp)	**378**, 381	**402**, 408	**399**, 405, [501]	**453**, 450	**606**, 585
Length (amino acids)	**125**, 126	**133**, 135	**131**, 134, [166]	**150**,149	**201**, 194
Sig.Cl[a]	**18–19**, 15–16	**19**–**20**	**18**–**19**	**41**–**42**	**18**–**19**
GPI[b]	**99**, 100	**(104)**, 108	((**97**)), ((104)), [((136))]		(((**183**)))
MW (nat.)[c]	**10–11**, ~11	~**11**, ~11.5	~11, [~14.5]	~**11**	**18–19**, ~18
MW (exp.)[d]	**13-14**	**13-14**	13–14, [16–17]	**12**–**13**	**20-21**, 19–20
MW (gel)[e]	**18–26**, 18–33	**10–19, 24**, 11–30	[15–20]	**15**, 10,13	**2x24**, 2x27
MW (gel,nr.)[f]	**18–26**, 36–49	**20–37**, 22–40	[15–20]	**32–36**, 2x22, 3x30–36	**37**, 2x27
N-glycosylation[g]	**N77**, N78, **N88**, N89			**N96**, N95	N63, **N141**, **N183**, N182
O-glycosylation[g]	**T105**, T106	S58, S66, T68			**(S114)**
Ser-P[h]	**S24**, S25, **S38**, S39	**S30**, **S33**, **S34**, S58, **S84**, S89, S114	**S22**, **S59**, S124	**S97**, **S98**, **S100**, **S105**, **S109**, **S114**, S121, **S122**, **S125**, S147	**S42**, **S44**, **S53**, S78, **S115**, S119, S122, S131, S170
Thr-P[h]	T21, **T67**, T68, **T79**, T91, **T105**, T106	**T40**, **T41**, T97	**T44**, **T58**	T43, T82, **T83**	T64, **T87**, T115, **T197**
Tyr-P[h]				**Y59**	T92, T103
PKC-P[h]	**S38**, S39, **T79**	**S34**, T97	**S59**, [S92], S124	*S12*	**S44**, T65, **S78**, T115
CK2-P[h]	**S67**, T68, T91	**S34**, **T41**, **S84**, S89		S98, T43	**S42**, **S115**, **S153**, S170, **S171**
CAMP-P[h]				**S97**, S147	**T87**
N-myristylation[i]	**G84**, G85	**G7**, **G15**, **G28**, **G29**, **114**	G53	G20, G72, **G73**, G76, **G77**	**G39**, **G43**, **G74**, G121, G125, G168, **G169**
C-lectin[j]				**C62-C89**, 61–68	

Data for the human proteins are shown in bold; data for the mouse proteins, underlined. Both bold and underlines represent the same data for the human and mouse proteins. (*) For Ly6G6e, the translated cDNA (399 bp) was used; for the mouse, the Ly6g6e5 form was used (indicated with square brackets).
[a] Signal peptide cleavage site.
[b] Likely site of GPI anchor attachment. Parentheses indicate decreased likelihood of GPI anchor attachment. More parentheses indicate less likelihood.
[c] Expected molecular weight (in kDa) of the fully processed (signal peptide cleaved and GPI anchor added if predicted) native protein.
[d] Expected molecular weight (in kDa) of the processed T7-tagged expression protein.
[e] Molecular weight observed on gel by Western blot analysis.
[f] Molecular weight observed on gel by Western blot analysis in nonreducing conditions.
[g] Types of glycosylation (*N* or *O*).
[h] Potential phosphorylation sites or potential sites of phosphorylation by those kinases.
[i] Potential N-terminal myristylation sites (in most of these cases unlikely, as the glycine is not the N-terminal residue).
[j] Potential C-lectin domain.

sizes by Western blot analysis (Fig. 2), consistent with the presence of PTMs. To examine this, we performed glycosidase treatments, and we observed that, in every case, both the human and its mouse ortholog showed the same pattern of glycosylation (Fig. 3). Human and mouse Ly6G6c, Ly6G5c, and Ly6G5b, and mouse Ly6g6d showed *N*-glycosylation, consistent with predictions. Ly6G6d and mouse Ly6g6e5 showed *O*-glycosylation and sialic acid residue modification, and in both cases the major difference in molecular weight was due to the addition of sialic acid residues to the *O*-glycan (Fig. 3).

To determine whether these Ly-6 proteins were GPI-anchored, the cells were treated with PI-PLC. LY6G6C, LY6G6D, and Ly6g6e5 all showed a decrease in detection in cells after treatment with PI-PLC, consistent with their release from the cell surface, indicating that these proteins are GPI-anchored (Fig. 4). LY6G5B, on the other hand, showed no change, even at the highest concentration of PI-PLC, indicating that this protein is not GPI-anchored and that there was no spurious degradation of the other MHC class III region Ly-6 proteins under the conditions used for the PI-PLC treatment.

To observe the subcellular localization of these Ly-6 proteins, their pattern of expression in the cell was investigated by immunofluorescence confocal microscopy under permeabilized and nonpermeabilized conditions. In every case, both the human and its mouse ortholog showed similar patterns of expression, with the same subcellular localization (Fig. 5). Ly6G6c, Ly6G6d, and mouse Ly6g6e4 and Ly6g6e5 all showed an extracellular cell surface pattern of expression, in agreement with the prediction that they were GPI-anchored cell surface proteins. All of the proteins showed very bright staining, though both mouse Ly6g6e constructs showed a decreased efficiency of transfection compared to the other constructs. Interestingly, all cell surface proteins showed

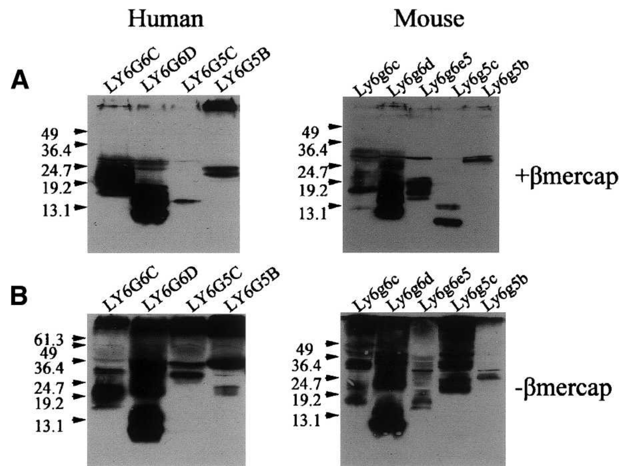

Figure 2. Dimer/multimer formation of the human and mouse MHC class III region Ly-6 proteins. Human (*left* panels) and mouse (*right* panels) proteins are shown. Expression was analyzed by SDS-PAGE under reducing (*A*) and nonreducing (*B*) conditions followed by Western blot immunostaining. Molecular markers are indicated in kilodaltons.

staining of filopodia (Fig. 5), long thin extensions of the plasma membrane normally involved in cell adhesion and migration (Wood and Martin 2002). Ly6G5c and Ly6G5b, on the other hand, showed no cell surface staining, consistent with the prediction and finding that they are not GPI-anchored proteins. In addition, in permeabilized conditions, they showed an intracellular staining pattern similar to that of ER staining, although it was a much more punctate staining pattern than that of the ER positive control, LPAAT (Aguado and Campbell 1998; data not shown).

FACS analysis

Fluorescence-activated cell sorting (FACS) analysis was used to identify cell types that express potential ligands and interaction partners for these human MHC class III region Ly-6 family members, by screening a variety of different cell lines. Two versions of the LY6G5B protein, one containing just the Ly-6 domain (LY6G5BL) (similar to the other constructs) and one containing the Ly-6 domain and the C-terminal region (LY6G5BS) were used in the screen. K562 cells (undifferentiated, erythrocyte/megakaryocyte precursor-like cell) seemed to express the potential ligands, with a very strong signal for LY6G6C, LY6G6D, LY6G5C, and Ly6g6e4 (Fig. 6; Table 3), and a very weak increase for LY6G5BL and mLy6g6e5. Raji (B cell) showed an increase in fluorescence only in the presence of LY6G6C, and a weaker increase with LY6G6D and LY6G5C (Table 3). U937 (macrophage), HeLa (epithelial), and MRC-5 (fetal lung) all showed high background fluorescence in the presence of the fluorescently-labeled antibody and the Fc protein control. However, an increase in fluorescence above this background level was observed in U937 in the presence of the LY6G6C and LY6G5C fusion proteins, indicative of the expression of a ligand or ligands on U937 for these

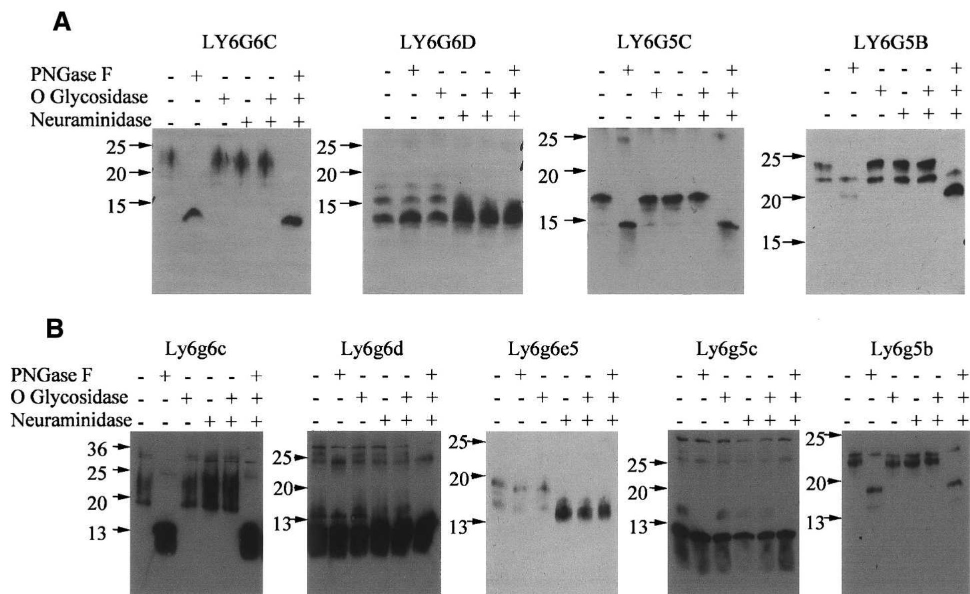

Figure 3. Glycosidase treatment of epitope-tagged human and mouse MHC class III region Ly-6 proteins expressed in Cos-7 cells. Cell lysates were treated with glycosidases alone or in combination, as indicated and analyzed by Western blot immunostaining. The human proteins (*A*) and mouse proteins (*B*) are shown. Molecular markers are indicated in kilodaltons.

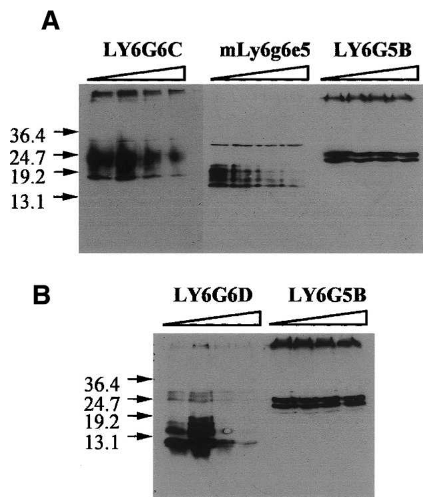

Figure 4. PI-PLC treatment of epitope-tagged human (and mouse) MHC class III region Ly-6 proteins expressed in Cos-7 cells. Cells expressing the T7 epitope-tagged Ly-6 proteins were treated with 0–0.005 U (*A*) or 0–0.02 U (*B*) PI-PLC. Cell lysates were analyzed by Western blot immunostaining. Molecular markers are indicated in kilodaltons.

proteins. Jurkat (T cell), Molt4 (T cell), and Hek293T (fetal kidney) cells were all negative (Table 3).

Discussion

To characterize the MHC class III region Ly-6 genes, the expression, localization, and PTMs of these proteins were determined. We have detected, by immunofluorescence analysis, that human and mouse Ly6G6c and Ly6G6d, and both mouse Ly6g6e4 and Ly6g6e5 are all cell surface molecules. These proteins were found to be highly expressed on filopodia. Filopodia, microfilopodia, or microspikes, presented by cells at their leading edges, are thought to be one of the cells' main sensory tools (Dalby et al. 2004). Filopodia are highly motile structures and are able to extend and retract as well as sweep up and down and from side to side while they search for their particular substrate, cell, or diffusible target molecule. Filopodia are used by many cell types as a sensing organ to explore the extracellular matrix and surface of other cells (Wood and Martin 2002) and have been associated with the sensing of chemoattractant gradients (haptotaxis) (Dalby et al. 2004 and references therein). When they have found their target, these filopodia are able to form adhesion junctions between themselves and the target cells. It has been described that filopodia are very delicate and do not appear to survive fixation well (Wood and Martin 2002). However, we have detected these filopodia very clearly on cells overexpressing human and mouse Ly6G6c and Ly6G6d, as well as mouse Ly6g6e4 and Ly6g6e5.

We found a clear correlation between the brightness of the staining patterns by immunofluorescence and the expression levels as detected by Western blotting. Both human and mouse Ly6G6c and Ly6G6d were well expressed, while Ly6G5c and Ly6G5b had much lower expression levels in Cos-7 cells. This is interesting, as all proteins were cloned using the same strategy, suggesting that there is something within the transcribed mRNA or the encoded protein that influences the expression level or stability of the protein.

Ly6G6c in both species and Ly6g6e5 and Ly6g5b in mouse appear to exist as monomers, while both human and mouse Ly6G6d and Ly6G5c and human LY6G5B appear to form larger complexes. The ability of these proteins to form part of larger complexes could be functionally significant. In the case of Ly6G5c and Ly6G5b, they might need to associate with an accessory protein for transport to the cell surface and be secreted. This accessory protein may not be well expressed in Cos-7 cells, explaining why it was not possible to detect these Ly-6 proteins in the cell supernatants as secreted proteins. This could also explain why mouse Ly6g5b is detected as a monomer because the murine protein does not bind to the monkey accessory protein. Secreted Ly-6 proteins isolated from rat urine were also described as not being highly expressed relative to other rat urinary proteins (Southan et al. 2002).

It is interesting to note that there are only ~27 Ly-6 family members described in human and 37 in mouse and that of those described, five are located in a cluster in the MHC class III region. In addition, only four human Ly-6 family members are known to be (or are potentially) secreted, and two of these are in this cluster (Ly6G5b and Ly6G5c) and are separated in the genome from the three GPI-anchored Ly-6 genes (*Ly6G6c*, *Ly6G6d*, and *Ly6G6e*) by the unrelated genes *G6F* and *BAT5* (Xie et al. 2003), indicating that there are two subclusters of these Ly-6 proteins in the class III region of the MHC. On human chromosome 8 (8q24-qter) a cluster of seven Ly-6 family members has been identified, and on mouse chromosome 15, syntenic to human chromosome 8q24 (Adermann et al. 1999; Stroncek et al. 2004), there is a cluster of nine Ly-6 family members. Another cluster with three members is found on human chromosome 19q13.

Some studies have shown that most hematopoietic cells express one or more members of the Ly-6 superfamily and that expression of Ly-6 proteins varies with hematopoietic cell development and activation (for review, see Bamezai 2004). However, the ligands for the Ly-6 members are poorly characterized (Pflugh et al. 2000, and references therein). A ligand for Ly-6d has been identified as a 9-kDa cell surface protein, which appears to be expressed widely (Apostolopoulos et al. 2000). Pflugh et al. (2000) have shown by flow cytometry assays

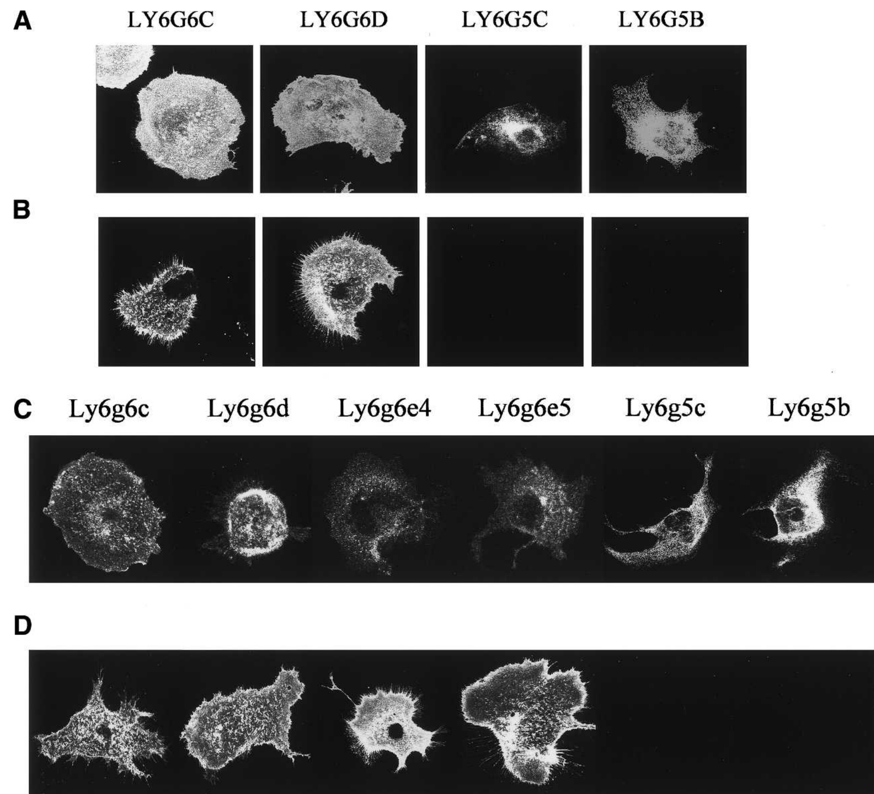

Figure 5. Immunofluorescence of epitope-tagged human (*A*,*B*) and mouse (*C*,*D*) MHC class III region Ly-6 proteins expressed in Cos-7 cells. The staining was performed under permeabilized conditions (*A*,*C*) and nonpermeabilized conditions (*B*,*D*).

that Ly-6A/E, Ly-6C, and Ly-6I recognize two potential ligands expressed by B lymphocytes. Studies by English et al. (2000) using a monoclonal antibody that inhibits Ly-6A/E-dependent cell–cell adhesion have identified a 66-kDa protein expressed in the thymus, spleen, B cells, and macrophages as a candidate ligand for Ly-6A/E.

When we searched for potential ligands of the MHC-encoded Ly-6 proteins by FACS analysis, we observed that the potential ligand(s) for LY6G6C, LY6G6D, LY6G5C, and Ly6g6e4 were mainly expressed on the undifferentiated megakaryocyte-like cell line K562. This restricted pattern of expression of the ligands could suggest that the signaling events mediated by these receptors play distinct roles in hematopoietic cell differentiation, with differentiated cells expressing a smaller range of ligands than undifferentiated cells. Alternatively, it could imply that differentiated cells need to be stimulated in some way before the ligands of these Ly-6 proteins are expressed.

Table 3. *Summary of FACS analysis results*

	Fc fusion construct									
Cell line	Cells	FITC	Fc	G6C	G6D	G5C	G5BL	G5BS	mg6e4	mg6e5
K562	0.33	8.82	14.9	**80**	**72.2**	**71.3**	36.7	18.1	**58**	37
U937	0.23	27.8	49.8	**77.1**	51.5	**68**	30.7	27.7	51.7	37.8
Molt4	0.03	0.11	0.23	0.07	0.16	0.12	0.23	0.26	0.2	0.11
Jurkat	0	0.24	0.14	0.16	0.06	0.22	0.3	0.11	0.14	0.11
Raji	0.2	0.41	0.37	**36.7**	**14.9**	**17.7**	0.62	0.37	1.4	3.17
HeLa	2.27	36.7	46.9	42.9	50.5	40.8	42.6	40.4	39.5	38.9
MRC-5	0.79	27	52.3	45.3	46.6	46.3	39.5	47.2	47.7	47.2
Hek293T	0.01	3	4.58	4.66	5.86	3.66	4.41	5.32	5.15	5.83

Positive results are highlighted in bold.

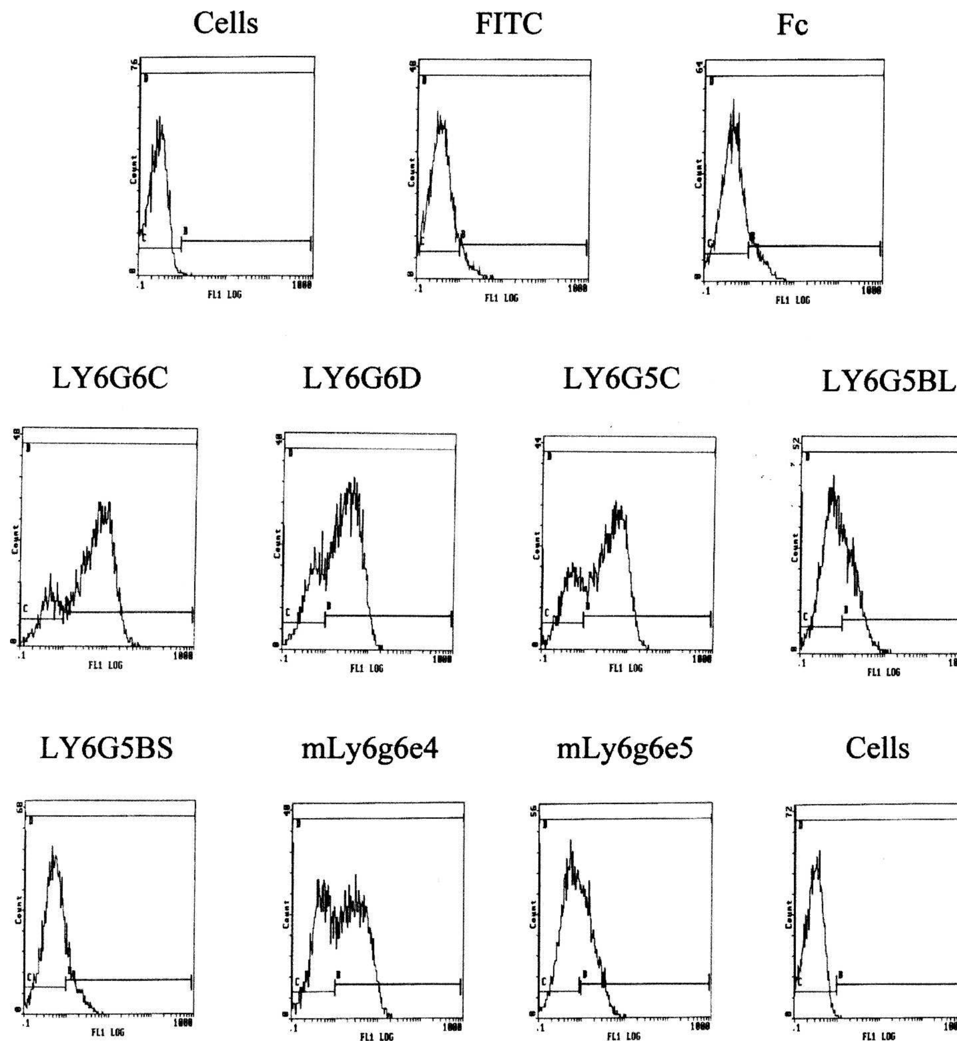

Figure 6. Expression of MHC class III region Ly-6 ligands on K562 cells. The highlighted area indicates the shift to increased fluorescence, showing clear positives.

Susceptibility to a significant number of human diseases, mostly autoimmune in nature, is associated with genes in the MHC. Recent studies using microsatellite and single nucleotide polymorphism (SNP) genotyping have attempted to fine-map the location of these genes. In particular, strong associations have been found between rheumatoid arthritis and a 126-kb region in the MHC class III region, between BAT2 and CLIC1, which includes the five Ly-6 members (Zanelli et al. 2001; Newton et al. 2004). In addition, alleles of the microsatellite marker D6S273, which lies in the *LY6G6D* gene, exhibit strong associations with IgA deficiency (Schroeder et al. 1998). The existence of SNPs in human LY6G6C, LY6G5C, and LY6G5B, which result in amino acid substitutions, as well as SNPs that lie in putative regulatory sequences have previously been described (Ribas et al. 2001). With our experimental data we could speculate immune-related roles for the MHC class III Ly-6 family members and that variation in expression of the genes and/or variation in the sequences of the encoded proteins could contribute to disease susceptibility.

In this study, we started from predicted Ly-6 protein sequences and their computational analyses. We performed experimental approaches to confirm those predictions, and our data have indicated that Ly6G6c, Ly6G6d, and mouse Ly6g6e are glycosylated, GPI-anchored, cell surface molecules, located on filopodia, which could act as cell receptors with potential roles in signaling or cell–cell interactions. Ly6G5c and Ly6G5b are glycosylated, potentially secreted proteins, which could act as ligands for other cell-surface receptors and could thus potentially have a role in cell signaling. The characterization of these five proteins is of great relevance, as they represent 18% of the human Ly-6 protein family and 50% of the secreted ones, and could help

us to understand the function of this and other members of the family by comparative analysis.

Materials and methods

Nomenclature note

To follow the gene nomenclature standards, all human genes are written in uppercase (e.g., *LY6G6C*), while mouse genes are written in lowercase with an initial capital letter (e.g., *Ly6g6c*). Orthologs in other species, or when referring to the genes in multiple species at once, are written in both upper- and lowercase (e.g., *Ly6G6c*).

Computational protein analysis

Protein sequence analysis was performed using the protein identification program PIX (http://www.rfcgr.mrc.ac.uk/Registered/Webapp/pix), and a variety of the proteomics tools available at the ExPASy site (http://www.expasy.ch), such as SignalP, PROSITE, NetOGlyc and NetPhos, and big-PI Predictor and DGPI. Homology searches of the five MHC class III region Ly-6 family members were performed using BLAST, comparing sequences against the NCBI nonredundant (nr) protein database. For human LY6G6E, the comparisons were with a translation of the 399-bp form found by RT-PCR (Mallya et al. 2002), but ignoring the premature termination codon, to translate to a theoretical full-length Ly-6 protein. For mouse Ly6g6e, the comparisons were done with the 405-bp form found by RT-PCR (Mallya et al. 2002), which would correspond to the orthologous sequence of the 399-bp human form, as for pig and rat Ly6G6e. Rat and pig orthologs were defined by BLAST comparisons against the available genomic sequences (accession nos. AC094348 and AL773591, respectively) but were not verified experimentally. The Blast 2 sequences program was used to compare MHC class III region Ly-6 proteins and their orthologs. Additionally, human CD59 (accession no. AAA88793) and mouse Ly-6A (accession no. NM_010738) were used for comparative analyses.

Creating N-terminal T7 epitope-tagged constructs (T7-Ly-6-pcDNA3)

The open reading frames of the human and mouse MHC class III region Ly-6 genes were PCR-amplified without the N-terminal signal peptide (exon 1) (Mallya et al. 2002) and cloned into an expression vector containing the human CD33 signal peptide followed by the T7-epitope tag (MASMTGGQQMGRPD) in pcDNA3 (see references in de Vet et al. 2001) to create N-terminal T7.Tag fusion proteins. Exons II and III (III and IV for *LY6G5CA*) (Mallya et al. 2002) were amplified with gene-specific primers containing a 5′ flanking BamHI site and a 3′ flanking XbaI site (Table 4). PCR conditions for all amplifications, using Platinum *Pfx* DNA polymerase (Life Technologies), were 94°C for 2 min; then 30 cycles of 94°C for 15 sec, 60°C for 30 sec, and 68°C for 45 sec, followed by 68°C for 5 min. The PCR buffer contained 1 mM $MgSO_4$, 1.2 mM dNTPs, and 0.3 μM each primer. The PCR products were digested and cloned directly into a CD33-T7-(BamHI-XbaI)-pcDNA3 vector (containing Kozak sequences; kindly donated by Dr. Edwin de Vet, RFCGR, Cambridge, UK) (de Vet et al. 2001). All the Ly-6 expression clones were sequenced using the T7 primer: 5′-TAATACGACTCACTATAGGG-3′, and the reverse primer SP6: 5′-TAGGTGACACTATAGAATAG-3′. As the human *LY6G6E* transcripts contained a premature termination codon (PTC) and would not translate to a functional protein (Mallya et al. 2002), only the sequences of the two mouse *Ly6g6e* transcripts were used. The 405-bp mouse Ly6g6e transcript contains an undisrupted Ly-6 domain (Ly6g6e4 construct), while the 501-bp mouse Ly6g6e transcript contains a disrupted Ly-6 domain (Ly6g6e5 construct) (Mallya et al. 2002). Sequence verified clones (human: T7-LY6G6C-pcDNA3, T7-LY6G6D-pcDNA3, T7-LY6G5C-pcDNA3, and T7-LY6G5B-pcDNA3; mouse: T7-Ly6g6c-pcDNA3, T7-Ly6g6d-pcDNA3, T7-Ly6g6e4-pcDNA3, T7-Ly6g6e5-pcDNA3,

Table 4. *Primers used for creating the human and mouse MHC class III region CD33-T7-Ly-6-pcDNA3 constructs*

Gene		Primer name	Primer sequence
Human			
LY6G6C	F	G6C2BHI	5′-CCGGGGATCCCGCTGACATTCGC-3′
	R	G6C3XBAI	5′-CCGGTCTAGATCAGTGCAGCAGCC-3′
LY6G6D	F	G6D2BHI	5′-CCGGGGATCCCGGAAACCGAATGC-3′
	R	G6D3XBAI	5′-CCGGTCTAGACTATCCGCTCCAC-3′
LY6G5C	F	G5C2BHI	5′-CCGGGGATCCCGGTAAGTTTGTTCC-3′
	R	G5C3XBAI	5′-CCGGTCTAGACTAAGGAGTATAGAG-3′
LY6G5B	F	G5B2BHI	5′-CCGGGGATCCCGTTCCTGTTCCC-3′
	R	G5B3XBAI	5′-CCGGTCTAGATCAGGAAGGGTGAGG-3′
Mouse			
Ly6g6c	F	mG6C2BHI	5′-CCGGGGATCCCGCTGATACTCGATG-3′
	R	mG6C3XBAI	5′-CCGGTCTAGATCAATGCAATAACCAGAG-3′
Ly6g6d	F	mG6D2BHI	5′-CCGGGGATCCCGGACACCGCACG-3′
	R	mG6D3XBAI	5′-CCGGTCTAGACTACAGTCCTGGCAAGAGCC-3′
Ly6g6e	F	mG6EBHI	5′-GGCCGGATCCCGGTCTCACCACTTCC-3′
	R	mG6EXBAI	5′-GGCCTCTAGACTAGAGGAAGTCATGTACTCC-3′
Ly6g5c	F	mG5C2BHI	5′-CCGGGGATCCCGGTGATACCAAGC-3′
	R	mG5C3XBAI	5′-CCGGTCTAGACTAGTGCATGCTATTCTTTC-3′
Ly6g5b	F	mG5B2BHI	5′-CCGGGGATCCCGCTCCAGTTGCC-3′
	R	mG5B3XBAI	5′-CCGGTCTAGATCAGGGTCTATCCCAGGG-3′

T7-Ly6g5c-pcDNA3 and T7-Ly6g5b-pcDNA3) were selected for further work.

Expression in Cos-7 cells

The human and mouse MHC class III region T7-Ly-6 proteins were expressed in Cos-7 (green monkey kidney) cells using the DEAE-dextran method as described elsewhere (de Vet et al. 2001). Three days after transfection, cells and supernatants were harvested. For direct analysis on 15% SDS-PAGE, cells were washed once with phosphate-buffered saline (PBS) and lysed in SDS-PAGE sample buffer (PSB) or nonreducing PSB (SDS-PAGE sample buffer without β-mercaptoethanol but with 0.2 mM iodoacetamide). Western blot immunostaining was performed with anti-T7 tag monoclonal antibody (mAb) (Novagen) and horseradish peroxidase (HRP)-coupled as secondary antibody, followed by detection with ECL (PerkinElmer Life Sciences). Immunofluorescence localization studies were performed as described elsewhere (Aguado and Campbell 1998), and staining was examined with a Nikon Eclipse E800 microscope linked to a MicroRadiance confocal imaging system (Bio-Rad).

Glycosidase treatment

The human and mouse MHC class III region T7-Ly-6 proteins expressed in Cos-7 cells were treated with *N*-glycosidase F, neuraminidase, or *O*-glycosidase as described elsewhere (de Vet et al. 2001), except that the samples were treated for 7 h at 37°C. Reactions were then stopped by the addition of PSB and analyzed on 15% SDS-PAGE followed by Western blotting. For mouse Ly6g5c, twice the number of cells were transfected, and for human LY6G5C, four times the number of cells, due to the low expression of these proteins.

Phospholipase C treatment

The human MHC class III region T7-Ly-6 proteins and mouse T7-Ly6g6e were expressed in Cos-7 cells, as described above. Three days post-transfection, the cells were washed on the plate with PI-PLC buffer (10 mM Tris-HCl, 144 mM NaCl at pH 7.4), and PI-PLC (Sigma) was added directly to the cells in the plate at a range of concentrations (0.0125 U/mL, 0.03125 U/mL, 0.0625 U/mL, 0.125 U/mL, and 0.25 U/mL) in PI-PLC buffer with 0.5% protease inhibitor cocktail and incubated with the enzyme at 30°C for 1 h. The supernatant was then collected and cleaned by centrifugation, and 0.5 μL of protease inhibitor was added to it. Cells were washed with PBS and then harvested in 105 μL of 1× PSB.

Immunoprecipitation

The human and mouse MHC class III region T7-Ly-6 proteins expressed in Cos-7 cells were lysed in immunoprecipitation (IP) lysis buffer (10 mM Tris-HCl at pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 0.02% NaN_3, and 1 mg/mL BSA) with 0.5% protease inhibitor cocktail. Lysates were cleared by centrifugation and immunoprecipitations performed with anti-T7 mAb and protein A-Sepharose (Sigma) at 4°C. The media were also immunoprecipitated after the addition of an equal volume of 2× IP lysis buffer. Proteins were eluted with PSB at 95°C for 2 min and analyzed by SDS-PAGE followed by Western blotting.

Creating C-terminally T7 and Fc-tagged soluble fusion proteins (Ly6-T7-pcD-sol and Ly6-Fc)

The Ly-6 domain, without the signal peptide or the C-terminal hydrophobic domain, of each of the human MHC class III region Ly-6 genes was PCR-amplified to create C-terminally T7-tagged soluble fusion proteins. For the *LY6G5B* gene, primers amplifying only the Ly-6 domain (LY6G5BL), as well as primers amplifying the Ly-6 domain and the C-terminal hydrophobic region (LY6G5BS) were used. PCR conditions were as described above using 1 μL of a 1 in 100 (v/v) dilution of each human Ly-6-pcDNA3 clone and mouse Ly6g6e4 and Ly6g6e5 clones as template.

For human *LY6G6C*, *LY6G6D*, *LY6G5C*, *LY6G5BL* and mouse *Ly6g6e4*, gene-specific forward and reverse primers both containing flanking NcoI sites were used (Table 5). These primers were designed to introduce a four–amino acid linker before the T7.Tag sequence in order for the Tag not to be masked. The PCR products were cloned into a CD33-(NcoI)-T7-pBLS vector. Clones were sequence-verified. To introduce a Kozak sequence at the 5′ end of the construct and a stop codon after the T7.Tag

Table 5. *Primers used for creating the soluble MHC class III region Ly-6 fusion constructs (Ly-6-T7-pcD-sol)*

Gene		Primer name	Primer sequence
LY6G6C	F	G6C2NCOI	5′-CCGGCCATGGCTGACATTCGCTGTC-3′
	R	G6C3NCOI	5′-CCGGCCATGGGTCCTGCGCTGTTG-3′
LY6G6D	F	G6D2NCOI	5′-CCGGCCATGGGAAACCGAATGCGG-3′
	R	G6D3NCOI	5′-CCGGCCATGGGCACGGCGCTGTTG-3′
LY6G5C	F	G5C2NCOI	5′-CCGGCCATGGGTAAGTTTGTTCCTGTC-3′
	R	G5C3NCOI	5′-CCGGCCATGGGTTGAGGGTCATTGC-3′
LY6G5BL	F	G5B2NCOI	5′-CCGGCCATGGTTCCTGTTCCCGAC-3′
	R	G5B3NCOI	5′-CCGGCCATGGGTGACCAGGAGTTGC-3′
LY6G5BS	F	G5B2ECORI	5′-GGCCGAATTCGTTCCTGTTCCCGAC-3′
	R	G5B3ECORI	5′-GGCCGAATTCGGAAGGGTGAGGTG-3′
Ly6g6e4	F	MG6E2NCOI	5′-CCGGCCATGGGTCTCACCACTTCCCC-3′
	R	MG6E3NCOI	5′-CCGGCCATGGGGGCGGCAGCATTG-3′
Ly6g6e5	F	MG6E2ECORI	5′-GGCCGAATTCGGTCTCACCACTTCCCC-3′
	R	MG6E3ECORI	5′-GGCCGAATTCATTGCATAGGTCCTGCTCAC-3′

sequence, the correct clones were PCR-amplified using the HindIIICD33 forward primer, 5′-GGCCAAGCTTACCATG CCGCTGCTG-3′, and the T7BamHI reverse primer, 5′-GATC TATGGATCCCGACCC-3′, respectively. The PCR products were cloned into a HindIII-BamHI-T7-pcDNA3 vector. For *LY6G5BS* and *Ly6g6e5*, as they contain NcoI restriction sites, a different cloning strategy was devised. Gene-specific forward and reverse primers containing flanking EcoRI sites were used (Table 5). To create a Kozak sequence at the 5′ end of the construct, the HindIIICD33 forward primer and the CD33EcoRI reverse primer (5′-GGCCGAATTCGGTATCCATAGCCAGGGC-3′) were used. The PCR products were digested with either HindIII and EcoRI (CD33) or EcoRI (*LY6G5BS* and *Ly6g6e5*) and cloned directly into a HindIII-EcoRI-T7-pcDNA3 vector. This strategy also introduced five additional amino acids before the T7.Tag sequence. All Ly-6-pcD-sol clones were sequenced using the T7 and SP6 primers, and the correct clones containing the Kozak sequence were isolated and used for further work (Ly6-T7-pcD-sol: human: LY6G6C-pcD-sol, LY6G6D-pcD-sol, LY6G5C-pcD-sol, LY6G5BL -pcD-sol, and LY6G5BS-pcD-sol; mouse: Ly6g6e4-pcD-sol and Ly6g6e5-pcD-sol).

These Ly-6 family members with an N-terminal CD33 signal sequence and a C-terminal T7.Tag were cloned into the pIGplus expression vector (Novagen) to create fusion proteins with the addition of the C terminus of the Fc portion of human IgG1. The Ly6-pcD-sol clones were digested and cloned directly into the HindIII-BamHI-pIGplus vector. Clones were sequenced using gene-specific primers and the correct clones identified (Ly6-Fc: LY6G6C-Fc, LY6G6D-Fc, LY6G5C-Fc, LY6G5BL-Fc LY6G5BS-Fc, Ly6g6e4-Fc and Ly6g6e5-Fc).

FACS analysis

Soluble Ly6-Fc fusion proteins (Ly6-Fc) and Fc as control were transiently expressed in Cos-7 cells, the media were harvested and centrifuged for 1 min at 13,000 rpm, and the supernatant was transferred to a fresh tube. Depending on the cell line, 0.5–1 mL ($1.5–3.5 \times 10^5$) cells were used in each analysis. The K562, U937, Molt4, Jurkat, Raji, Hela, MRC-5, Hek293T, and Cos-7 cell lines were analyzed. Cells were pelleted (2000 rpm) for 5 min at room temperature, resuspended in 250–500 μL of Cos-7 cell media containing the expressed Ly6-Fc fusion protein, and incubated on ice for 45 min. Cells were then centrifuged for 5 min at 4°C at 2000 rpm, resuspended in 50 μL of a 1 in 100 dilution (v/v) of anti-human IgG-FITC conjugated antibody in 0.2 mg/mL BSA/PBS, and incubated on ice for 45 min. One milliliter of cold 0.2 mg/mL BSA/PBS was added, and the cells were centrifuged for 5 min at 4°C at 2000 rpm. Cells were then resuspended in 500 μL of 0.5% formaldehyde/PBS, and the fluorescence was measured. The negative controls were as follows: cells resuspended in 250 μL of media expressing only the Fc protein or only BSA/PBS and incubated with anti-human IgG-FITC conjugated antibody, or only fresh cells resuspended in 0.5% formaldehyde/PBS, tested before and after the experiment. FACS analysis was performed on a Beckman Coulter EPICS XL-MCL FACS machine.

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