The cation-independent mannose-6-phosphate receptor binds to soluble GPI-linked proteins via mannose-6-phosphate

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Abstract The cation-independent mannose-6-phosphate/insulin-like growth factor II receptor has been observed to bind to soluble forms of glycosyl-phosphatidylinositol-linked molecules, one of mammalian origin (rat Thy-1) and two of protozoan origins. Of the two phosphate groups found on the soluble forms of the protozoan glycosyl-phosphatidylinositol-linked molecules: (i) the internal mannose-6-phosphate diester (which forms a part of the ethanolamine bridge) and (ii) the inositol-1,2 cyclic phosphate group (which arises after cleavage of the membrane associated form with phosphatidylinositol-specific phospholipase C), only the former appears to be recognized by the mannose-6-phosphate/insulin-like growth factor II receptor, as mild acid hydrolysis which destroys the latter has been observed not to affect the receptor binding site.

Key words: Mannose-6-phosphate/insulin-like growth factor II receptor; Glycosyl-phosphatidylinositol-linked molecule; Phosphatidylinositol-specific phospholipase C; Cell growth regulation; Carbohydrate recognition

1. Introduction

The cation-independent mannose-6-phosphate receptor is a type I transmembrane protein involved in the targeting of newly synthesized, mannose-6-phosphorylated lysosomal enzymes to lysosomes (for review see [1,2]). This receptor has been shown to recycle between the prelysosomal/endosomal compartment and the cell surface, where it occurs in association with clathrin-coated pits [3,4]. Molecular cloning of the mannose-6-phosphate receptor [5] and the insulin-like growth factor II receptor [6] led to the conclusion that the two receptor functions are on the same protein [7–9], which is referred to here as the MPR. These findings raised the intriguing possibility [10] that the responses of this growth-regulatory molecule could be influenced by interactions with carbohydrates on neighbouring molecules, and lent weight to the proposal [11] that there may exist growth-regulating networks based on carbohydrate–protein interactions.

The extracellular domain of the MPR contains 15 contiguous domains with sequence identities of 16–38% [12]. Evidence has been presented that the binding sites for IGF-II and mannose-6-phosphate on the MPR are distinct [13,14] and that there are only two mannose-6-phosphate binding sites [15], one of which is located within domains 1–3 and the other, domains 7–10/11. Therefore, the majority of the 15 repeating domains of the MPR may not be involved in mannose-6-phosphate binding [16]. In the course of a search for additional saccharide ligands that may be recognized by these other domains of the MPR, we have performed binding studies (P.G. and T.F., unpublished observations) using a diverse array of structurally defined lipid-linked oligosaccharides, encompassing neutral and acidic oligosaccharide sequences that occur commonly on *N*- and *O*-glycosylated proteins, polysaccharides and glycolipids [17–19]. Among these binding was detected, as predicted, to the neoglycolipid of the 6-phosphorylated pentamannose oligosaccharide derived from *Hansenula holstii* phosphomannan [19,20]. Binding was also detected to the soluble form of variant surface glycoprotein (sVSG) from *Trypanosoma brucei*, which was generated by digestion of the glycosyl-phosphatidylinositol (GPI)-linked molecule by phosphatidylinositol-specific phospholipase C (PI-PLC).

GPI-linked molecules contain at least two phosphate groups (Fig. 1), one as part of the internal mannose-6-phosphate ethanolamine bridge; the other connects the glycan to the lipid moiety in intact GPI-linked molecules, or it is a part of an inositol-1,2 cyclic phosphate group in GPI-linked molecules that have been cleaved with PI-PLC. In view of the role of the MPR as a growth-regulatory molecule and the observation that under certain circumstances GPI-linked molecules may mediate cell signalling [21], we have pursued further our initial observation on the binding of the receptor to a GPI-linked molecule. In particular, our aim has been to determine which of these two phosphate groups (P1 or P2, in Fig. 1) is involved in the recognition by the MPR.

2. Materials and methods

2.1. Cation-independent mannose-6-phosphate/insulin-like growth factor II receptor

A mono-disperse preparation of the 205 kDa, cation-independent mannose-6-phosphate/insulin-like growth factor II receptor (MPR) was made from fetal calf serum using a phosphomannosyl ester (PPME) adsorbent [22]. For radioiodination using iodogen from Sigma Chemical Co., Poole, Dorset, UK [23], 50 μg of the protein (estimated

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Abbreviations: BSA, bovine serum albumin; ECL, enhanced chemiluminescence; GPI, glycosyl-phosphatidylinositol; IGF-II, insulin-like growth factor II; MPR, cation-independent mannose-6-phosphate/insulin-like growth factor II receptor; mf, membrane form of GPI-anchored protein; PBS, phosphate-buffered saline (10 mM phosphate buffer in 140 mM NaCl pH 7.4); PI-PLC, phosphatidylinositol-specific phospholipase C; PPME, phosphomannosyl ester; s, soluble form of GPI-anchored protein; TBS, Tris-buffered saline (10 mM Tris-HCl in 140 mM NaCl); VSG, variant surface glycoprotein.

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using the Bio-Rad Protein Assay Dye Reagent Concentrate with bovine serum albumin (BSA) as the standard) was precipitated in 80% (v/v) acetone to remove sodium chloride and sodium azide preservative.

2.2. GPI-anchored proteins

GPI-anchored proteins were prepared as follows, in their intact membrane forms (mf) lacking the cross-reacting determinant (CRD) or as PI-PLC cleaved, soluble forms (s) which express the CRD. The soluble form of VSG (sVSG), variant MITat1.4, was purified from bloodstream form *Trypanosoma brucei* as described previously [24]. Whole *T. brucei* (variant MITat1.4) cell lysates (5×10^8 cells/ml) containing the membrane form of the VSG (mfVSG) or sVSG were prepared as described in [25]. In some experiments, mfVSG was converted to sVSG in situ, after electro-transfer onto nitrocellulose (as described below), by treating the nitrocellulose membranes with a recombinant *Bacillus thuringiensis* PI-PLC (Oxford Glycosystems, Abingdon, Oxon, UK), 2 IU/ml in phosphate-buffered saline (PBS) for 1 h at 20°C. In other experiments, purified sVSG was subjected to mild acid hydrolysis in situ, after electro-transfer onto nitrocellulose, by soaking the nitrocellulose membranes in 1 M HCl for 1 h at 20°C [25]. In both cases, nitrocellulose membranes were washed three times in PBS prior to further processing.

The membrane form of *Leishmania major* Gp63 (mfGp63, also known as promastigote surface protease) was purified as in [26] and was a gift from Dr. Pascal Schneider. The soluble form of Gp63 (sGp63) was prepared by treating mfGp63 with recombinant *B. thuringiensis* PI-PLC (Oxford Glycosystems) for 4 h at 37°C in Tris-buffered saline (TBS), pH 8.0 using 0.5 IU of enzyme per 5 μg of protein in a total volume of 10–20 μl.

The membrane form of rat brain Thy-1 antigen (mfThy-1) was a gift of Dr. Neil Barclay. The soluble form of Thy-1 (sThy-1) was prepared by treating mfThy-1 with recombinant *B. thuringiensis* PI-PLC, as described above.

2.3. Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), was performed [27] using 6% (w/v) polyacrylamide under non-reducing conditions for MPR, and 12% (w/v) polyacrylamide under reducing conditions for the GPI-anchored proteins.

2.4. Binding assays

Binding of ^{125}I-labelled MPR was evaluated to GPI-anchored proteins after SDS-PAGE and electro-transfer onto nitrocellulose [28], using a 1 μl spot (1 μg) of PPME as a positive control. Prior to overlaying with the radioiodinated protein, the blots were soaked for 2 h at 20°C in 3% (w/v) BSA in PBS. The nitrocellulose was overlaid for 2 h at 20°C with [^{125}I]MPR (5×10^5 cpm/ml) in the absence or presence of various inhibitors as indicated in the figure legends. The nitrocellulose was washed in PBS, dried and radiobinding was detected by autoradiography. Replicate lanes were stained with amido-black to reveal electro-transferred proteins.

For detecting the CRD on GPI-anchored proteins that have been cleaved by PI-PLC (in solution (Gp63 and Thy-1) or on nitrocellulose membranes (VSG)), the electro-transferred proteins, treated with 3% (w/v) BSA in PBS on the membranes, were overlaid with anti-CRD (polyclonal rabbit IgG antibodies (1.5 μg/ml) which were raised against a different sVSG variant (ILTat1.21) and absorbed with mfVSG, from Oxford Glycosystems), and washed according to the manufacturer's instructions. Bound antibodies were detected with anti-rabbit IgG conjugated to horseradish peroxidase (Sigma), 1:1000 dilution, for 1 h at 20°C. After three washes the nitrocellulose was subjected to enhanced chemiluminescence, ECL (Amersham International plc., Little Chalfont, Bucks, UK) according to the manufacturer's instructions.

3. Results and discussion

In binding experiments using the radioiodinated monodisperse MPR (Fig. 2) an interaction was observed with sVSG which had been electro-transferred onto nitrocellulose (Fig. 3, lane 1). The binding appeared to have a mannose-6-phosphate

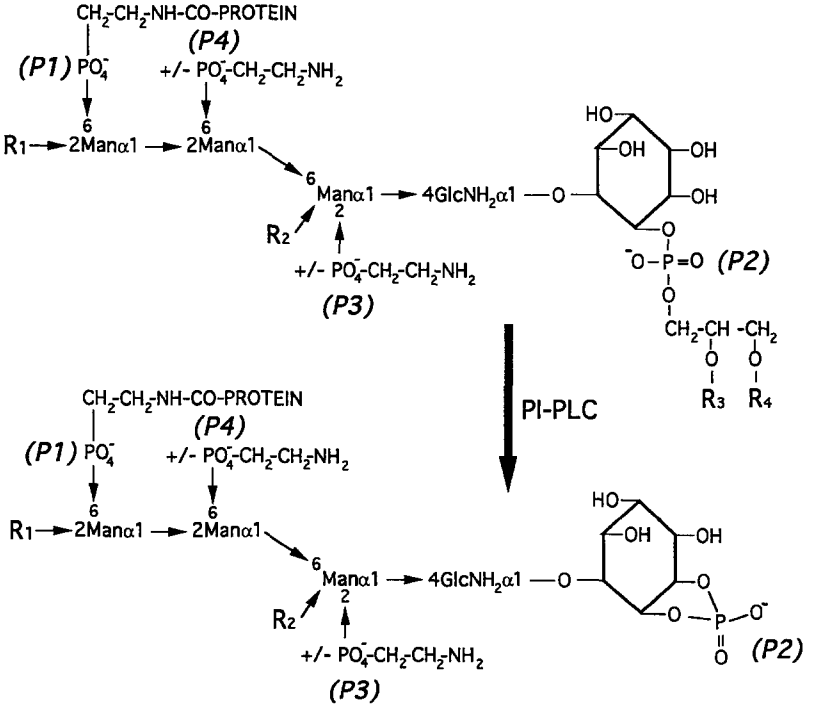

Fig. 1. The consensus structure of a GPI-anchor is shown (top) together with the product of PI-PLC digestion (bottom). All GPI-anchors contain an ethanolamine phosphate bridge (P1), in mannose-6-phosphate diester linkage, and a phosphate group (P2) linking the inositol to the lipid anchor. Protozoal GPI-anchors, such as those of *T. brucei* VSG and *L. major* Gp63, do not contain additional ethanolamine phosphate groups. Mammalian GPI-anchors generally contain second ethanolamine phosphate group (P3) in a mannose-2-phosphate diester linkage, and may also contain an additional ethanolamine phosphate group (P4) in a mannose-6-phosphate diester linkage. The groups R_1 and R_2 are variable carbohydrate substituents, R_3 is a fatty acyl chain and R_4 is either a fatty acyl chain or a fatty alkyl chain.

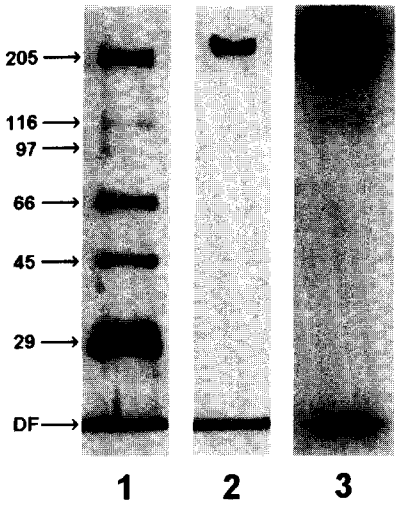

Fig. 2. SDS-polyacrylamide gel electrophoresis of MPR. The purification of MPR was as described in section 2. The protein was electrophoresed under non-reducing conditions in a 6% (w/v) polyacrylamide gel. Unlabelled proteins (lanes 1 and 2) were visualized using silver stain [48]; the radiolabelled MPR was visualized by autoradiography. Lane 1 shows molecular mass markers: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97 kDa), BSA (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa). DF = dye front of gel. Lane 2 contained the purified MPR (1 μg); lane 3, autoradiograph of [^{125}I]MPR (1×10^6 cpm).

specificity in accord with existing knowledge: like the binding to PPME, the binding to sVSG was inhibited in the presence of mannose-6-phosphate (Fig. 3, lane 2); the binding was not inhibited by galactose-6-phosphate, and there was no suggestion of specificity towards the monosaccharides mannose or galactose, both of which are present on this variant of VSG (Fig. 3, lanes 3–5).

The MPR bound to the soluble form of a second parasite-derived GPI-linked protein, Gp63 from *L. major* (Fig. 4A, lane 2′) and also to the soluble form of rat Thy-1, a mammalian GPI-linked protein (Fig. 4B, lane 4). Binding to both glycoproteins was inhibitable by mannose-6-phosphate (results not shown). With all three of the GPI-linked molecules investigated there was no binding of the MPR to the membrane forms; results for mfGp63 are in Fig. 4A, lane 1′, those for mfVSG and mfThy-1 are in Fig. 4B, lanes 1 and 3, respectively.

When soluble GPI-linked proteins are produced by PI-PLC cleavage, an inositol-1,2 cyclic phosphate group is generated (P2 in Fig. 1). The CRD is an obligatory component of an epitope common to all GPI-linked proteins cleaved by this method [29–33]. Thus, anti-CRD antibodies bind only to soluble forms and not to membrane forms of GPI-linked molecules, as shown for Gp63 in Fig. 4A, lanes 1 and 2. When mfVSG was converted to sVSG by PI-PLC treatment whilst immobilized on nitrocellulose, the CRD was generated (Fig. 4D, lane 1′), however, binding to MPR was not observed (Fig. 4D, lane 1). This suggests that mfVSG when cleaved by PI-PLC whilst on the nitrocellulose is in a suitable orientation to be bound by anti-CRD antibodies but not by the MPR. Second, the results suggest that the inositol-1,2 cyclic phosphate group is not the recognition site for the MPR, rather the determinant recognized is the internal mannose-6-phosphate diester. This would be in accord with previous observations that MPR binds to mannose-6-phosphate diesters that occur on lysosomal enzymes of the slime mould *Dictyostelium discoideum* [15,34]. This idea is supported by the results of an experiment in which sVSG was subjected to mild acid hydrolysis to convert the inositol-1,2 cyclic phosphate to inositol-1-phosphate and inositol-2-phosphate and, hence destroy the CRD, leaving the remainder of the sVSG intact [25]. Fig. 5 shows that even after successful destruction of the CRD (lanes 1′ and 2′), the MPR is able to bind to sVSG (lanes 1″ and 2″). Amido black protein staining showed that the sVSG remained on the nitrocellulose after the mild acid hydrolysis (lanes 1 and 2).

These results indicate that the recognition element for MPR on sVSG is the mannose-6-phosphate diester. The diester group is present in both soluble and membrane forms of GPI-linked molecules and the results of the PI-PLC cleavage experiment (Fig. 4D) indicate that the accessibility of this mannose-6-phosphate diester may be modulated by the orientation or mode of presentation of the glycoprotein. There is a precedent for this type of phenomenon: inherently alkali-labile fatty acid ester linkages on GPI-anchors are remarkably resistant to alkali when membrane form GPI-anchored proteins are electrotransferred to nitrocellulose [25]. Given that some highly-glycosylated GPI-anchored proteins bind exclusively via their lipid moiety [35] it seems likely that most membrane form GPI-proteins are oriented with their GPI anchors in contact with the nitrocellulose membrane. Assuming that this orientation is maintained after PI-PLC cleavage of mfVSG on the membrane (by adjacent protein nitrocellulose contacts), steric constraints may prevent the MPR from reaching the mannose-6-phosphate diester in this case. In contrast, sVSG is likely to bind in a relatively random fashion and therefore present most of the mannose-6-phosphate diester groups in a less constrained manner. Thus, although we were unable to demonstrate an interaction between MPR and membrane form GPI-linked molecules when immobilized on nitrocellulose membranes, it remains possible that under certain circumstances in vivo, the membrane forms of GPI-linked molecules are accessible to the MPR.

It should be noted that while protozoal GPI-proteins have been used here to distinguish the relative contributions of mannose-6-phosphate diester and inositol-1,2 cyclic phosphate, all

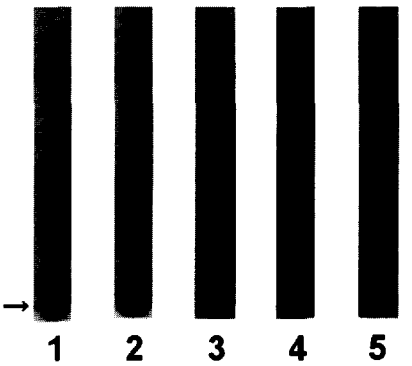

Fig. 3. Binding of [^{125}I]MPR to purified sVSG in the absence and presence of inhibitors. sVSG (5 μg) was electrophoresed under reducing conditions in a 12% (w/v) SDS-polyacrylamide gel, electro-transferred onto nitrocellulose and overlaid with [^{125}I]MPR (5×10^5 cpm/ml) in the absence (lane 1) or presence of the following inhibitors (all at 10 mM): mannose-6-phosphate (lane 2), galactose-6-phosphate (lane 3), mannose (lane 4) and galactose (lane 5). Binding was detected by autoradiography. A 1 μg spot of PPME (arrowed) was placed onto the nitrocellulose prior to being overlaid with [^{125}I]MPR, as a positive control.

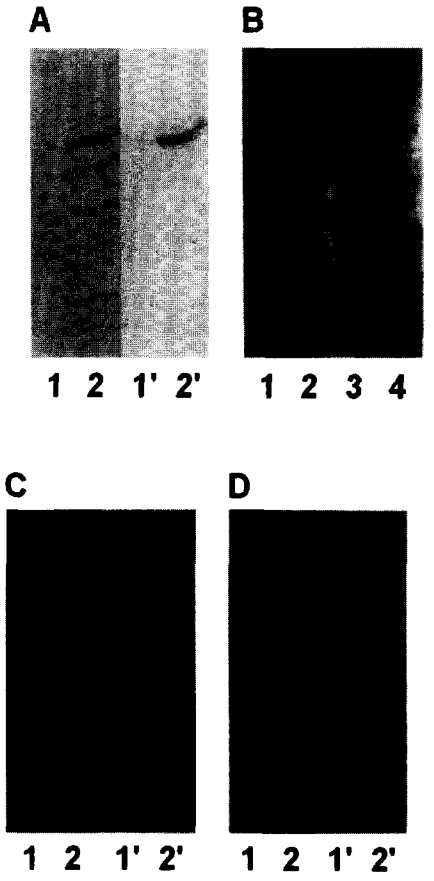

Fig. 4. Binding of [^{125}I]MPR to soluble GPI-linked molecules, but not to their membrane forms. Mf and s forms of Gp63, VSG (present in whole-cell lysates) and Thy-1 (5 μg each) were electrophoresed, transferred onto nitrocellulose and variously treated as follows. In panel A, mf and sGp63 lanes 1′ and 2′, respectively, were overlaid with [^{125}I]MPR and binding was detected by autoradiography; thereafter the same lanes (1 and 2) were overlaid with polyclonal anti-CRD antibodies, and binding detected using ECL. In panel B, mf and sVSG (lanes 1 and 2, respectively) and mf and sThy-1 (lanes 3 and 4, respectively) were overlaid with [^{125}I]MPR and binding detected by autoradiography. In panel C, which was a control experiment for panel D, mf and sVSG (lanes 1 and 2, respectively) were tested for binding to [^{125}I]MPR; duplicate lanes (1′ and 2′, respectively) were tested for binding to anti-CRD antibodies using ECL. In panel D, mf and sVSG (lanes 1 and 2, respectively) were treated on the nitrocellulose with PI-PLC as described in section 2 and tested for binding to [^{125}I]MPR; duplicate (lanes 1′ and 2′, respectively) were similarly treated with PI-PLC and tested for binding to anti-CRD antibodies. Note: the weak binding of the anti-CRD antibodies in panel C, lane 1′ is due to a small amount of sVSG in the cell lysate containing predominantly mfVSG. The anti-CRD positive bands seen below and above the sVSG band in panel C, lane 2′ and panel D, lane 2′ are due to some proteolysis and aggregation of the sVSG.

higher eukaryote GPI-anchors contain additional ethanolamine phosphate groups, reviewed in McConville and Ferguson [36] (see P3 and P4, Fig. 1). Thus all of the mammalian GPI-anchors contain a mannose-2-phosphate diester and many, e.g. human erythrocyte acetylcholinesterase [37] and human CD 52 (Truemann, Lifely, Schneider and Ferguson, unpublished data) contain an additional mannose-6-phosphate diester on a proportion of their GPI-anchors. While the mannose-2-phosphate diester is unlikely to be a ligand for the MPR, there is a possibility that some anchors (those bearing two mannose-6-phosphate diesters) could simultaneously ligate two MRP mannose-6-phosphate binding sites, in either an intermolecular or intramolecular manner. It has been postulated [38] that the well documented phenomenon of transmembrane signalling via the clustering of GPI-anchored proteins with antibodies and secondary antibodies may involve a transmembrane protein that can sense this clustering. Thus there is the possibility that, in cells that express MPR on their surface, such as the BW5147 T cell lymphoma [3] which is also responsive to GPI-mediated signals, clustered GPI-linked proteins could form networks with MPR molecules and that such a co-clustering of transmembrane MPR might be responsible for transmembrane signalling in some circumstances. Other transmembrane proteins have been suggested to associate with GPI-anchored proteins and, in some cases, to possibly mediate transmembrane signalling following the clustering of the GPI-anchored proteins. These include caveolin [39,40], the T-cell receptor/CD3 complex [41,42] and the complement receptor type 3 (CR3) [43,44]. Thus, while the physiological significance of transmembrane signalling via the artificial clustering of GPI-anchored proteins remains unclear, the transducing machinery may vary according to the GPI-protein in question and the cell type it is expressed in.

Experiments carried out using kidney basolateral membranes have shown that mannose-6-phosphate containing peptides may activate the MPR in the absence of insulin-like growth factor II (IGF-II) [45,46]. Others [47] have proposed that mannose-6-phosphate containing peptides per se do not have the ability to activate the MPR, and that activation of the receptor by IGF-II is blocked by occupancy of the mannose-6-phosphate binding sites. It is possible that this binding may uncouple a growth regulatory network by displacing mannose-6-phosphate containing endogenous proteins. Whatever the mechanism involved, there appears to be a role for mannose-6-phosphate as a modulator of IGF-II signal transduction in vivo. The observations described here on the interactions between the MPR and the mannose-6-phosphate diester of soluble GPI-linked molecules open the way to further investigations, and in particular as to whether this binding can modulate the function of the MPR as a growth regulator.

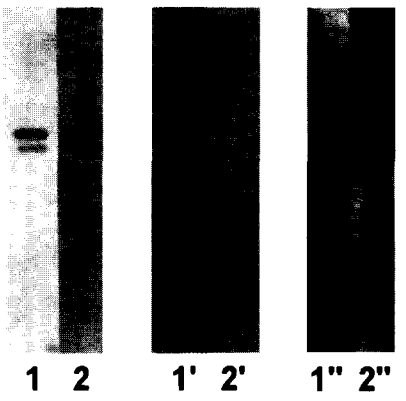

Fig. 5. Binding of [^{125}I]MPR to sVSG (present in whole-cell lysates) before and after acid hydrolysis to destroy the CRD. Multiple lanes of sVSG were electrophoresed and electro-transferred to nitrocellulose as described in Fig. 3. Native sVSG (lanes 1, 1′ and 1″) or sVSG mild acid treated on the nitrocellulose (lanes 2, 2′ and 2″) were stained with amido black (lanes 1 and 2) or overlaid with anti-CRD antibodies and developed using ECL (lanes 1′ and 2′) or overlaid with [^{125}I]MPR and binding detected by autoradiography (lanes 1″ and 2″).

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