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Published in:
British journal of haematology

DOI:
10.1046/j.1365-2141.1997.582706.x

Citation for published version (APA):
A phenylalanine-55 to serine amino-acid substitution in the human glycoprotein IX leucine-rich repeat is associated with Bernard-Soulier syndrome

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Received 2 October 1996; accepted for publication 4 February 1997

Summary. The platelet membrane glycoprotein (GP) Ib–IX–V complex, the major von Willebrand factor receptor on platelets, is absent or dysfunctional in patients with the Bernard-Soulier syndrome (BSS). The four single subunits of the GPIb–IX–V complex (GPIba, Ibβ, IX and V) are molecular products of different genes. Several point mutations and deletions affecting the GPIba gene have been identified as the cause of BSS, whilst in four BSS families a GPIX gene defect has been reported. Moreover, a single case of BSS has been associated with a genetic defect of GPIbβ. We investigated the molecular basis of another case of BSS with a deficient expression of GPIX, as detected by immunofluorescence studies. After amplification of the entire GPIX coding region, nucleotide sequence analysis showed a homozygous single point mutation predicting a phenylalanine to serine substitution at position 55 of the mature GPIX within its unique leucine-rich repeat. By allele-specific oligonucleotide hybridization we confirmed the homozygosity of the patient as well as the carrier state of two out of three of his children studied. Although the parents of the patient, who were first cousins, were no longer alive and thus not available for study, we speculate that the molecular defect observed in the proband was inherited from both parents, who probably were heterozygous for this GPIX gene defect.

This study confirms that BSS may be caused by many different subtle molecular defects that often prevent the assembly and expression of a functional GPIb–IX–V complex.

Keywords: platelet glycoproteins, Bernard-Soulier syndrome.

Bernard-Soulier syndrome is a rare congenital bleeding disorder characterized by mild to moderate thrombocytopenia, abnormally large platelets and a prolonged bleeding time. It is usually inherited in an autosomal recessive manner (Bernard & Soulier et al., 1948; Berndt et al., 1989).

Platelet functional studies show normal platelet aggregation in response to collagen, adenosine diphosphate and thrombin, but absent, or severely reduced, ristocetin-induced platelet agglutination, not reversed by the addition of normal plasma (Bithell et al., 1972). The defective platelet function in BSS patients is caused by the deficiency and/or the dysfunction of the platelet membrane GPIb–IX–V complex (Clemetson et al., 1991, 1982; Berndt et al., 1983; Nurden et al., 1983) that represents the major von Willebrand factor receptor on the platelet surface. The binding site for von Willebrand factor is located in its GPIba chain, between residues 220 and 310 (Titani et al., 1987; Handa et al., 1986). An optimal interaction between subendothelial von Willebrand factor and GPIb is crucial for platelet adhesion to exposed vascular subendothelium, notably under conditions of high shear rate (Weiss et al., 1978, 1989).

In cytoplasm, the GPIba carboxyl-terminal end is associated with actin-binding protein with actin filaments. Thus, the complex represents a major attachment site between platelet membrane and cytoskeleton, responsible for the shape of the platelets and their membrane stabilization (Fox, 1993). Its disruption may partially explain the increase in platelet volume in BSS.

Very recently, a high-affinity α-thrombin binding site has
been identified between amino-acids 269 to 287 in the GPIbα chain (Gralnick et al., 1994), in addition to the moderate-affinity seven-transmembrane platelet-thrombin receptor (Greco et al., 1996; Vu et al., 1991; Hung et al., 1992). Thus, the GPIb–IX–V complex also seems to play an important role in the initiation of thrombus formation.

The GPIb–IX–V complex consists of four associated glycoproteins. GPIbα (Mr 143,000) and GPIbβ (Mr 22,000) are disulphide bond in the GPIb heterodimer which is non-covalently bound to GPI (Mr 20,000) and GPV (Mr 81,000) (Roth, 1991; Modderman et al., 1992; Lopez et al., 1987, 1988; Hickey et al., 1989, 1993a). All four GPs are encoded by different genes and belong to the family of leucine-rich glycoproteins (LRG) characterized by the presence of leucine-rich motifs (Lanza et al., 1993; Wenger et al., 1988; Hickey & Roth et al., 1993; Yagi et al., 1994, 1995). The function of other members of this family is still not completely known, but all are supposed to be involved in cell–cell and protein–protein interactions (Roth, 1991). Whereas seven and 15 LRG repeats are present on GPIbα and on GPIV respectively, one unique motif is located in GPIbβ and GPIX molecules (Lopez et al., 1988; Hickey et al., 1989, 1993).

In BSS patients the expression of all GPIb–IX–V complex components can be variably affected: it is either absent or decreased, or sometimes a normal complement on the membrane of each subunit can be demonstrated among different pedigrees (Clemetson et al., 1982; Berndt et al., 1983; Ware et al., 1990; Miller et al., 1992).

Several different mutations leading to a BSS phenotype have been reported in the GPIbα gene (Ware et al., 1990, 1993; Miller et al., 1992; Simsek et al., 1994a, b; De La Salle et al., 1995; Li et al., 1995; Furuhata et al., 1994; Kunishima et al., 1994; Lanza et al., 1993; Margaglione et al., 1995; Kanaji et al., 1995; Yamamoto et al., 1995). On the contrary, a single case of BSS associated with a molecular defect in the GPIbβ gene has been reported (Butdarf et al., 1995). Until now, a defect of the GPIX gene has been shown to underlie this congenital bleeding disorder in four families (Wright et al., 1993; Clemetson et al., 1994; Noda et al., 1995a, b).

We now report an additional GPIX gene defect resulting in a BSS phenotype. Immunofluorescence studies performed in a Dutch patient with the Bernard-Soulier syndrome showed that GPIX was almost undetectable whereas the level of GPIbα and GPV was decreased. After genomic DNA amplification, we detected a homozygous missense mutation affecting the GPIX gene. Furthermore, both children of the patient available for study were shown to be carriers of the same GPIX molecular defect.

CASE REPORT

The patient, a 75-year-old male, has shown a lifelong bleeding tendency. Spontaneous epistaxis often occurred until the age of 20 after which the frequency decreased. He underwent splenectomy when he was 24 years old. After the age of 35 there were only gingival bleedings, with inconstant frequency and severity. A 6-week period characterized by melena occurred when he was 43. Hospitalization was necessary. The patient recovered after medical treatment and neither platelet nor red cell transfusions were needed. No other gastro-intestinal bleedings occurred in the following years.

Blood smear showed a prominent population of giant platelets: platelet aggregation was normal in response to collagen and adenosine diphosphate stimulation, but absent after ristocetin either in platelet-rich plasma or, after platelet washing, in the presence of normal plasma. Thrombocytopenia was always observed, ranging between 50 and 75 × 10⁹ platelets/l. The bleeding time performed by the modified Ivy method was between 4.5 and 6 min.

Both parents of the patient had died and therefore were not available for study, but they had no history of bleeding problems: they were first cousins. None of the three patient’s children showed a bleeding tendency.

Blood samples were collected from the patient, his available family members and two normal volunteers. All individuals gave their informed consent before blood drawing.

MATERIALS AND METHODS

Flow cytometry studies. Blood was collected by venepuncture and anticoagulated with EDTA. Platelets were isolated from platelet-rich plasma obtained by centrifugation of anticoagulated blood at 200 × g for 10 min.

Washed platelets were fixed in 1% paraformaldehyde solution for 5 min at room temperature. After three washes with phosphate-buffered saline/10 mM EDTA/0.2% wt/vol bovine serum albumin (PBS/EDTA/BSA), 6 × 10⁸ platelets were incubated with different monoclonal antibodies (MoAbs) for 30 min at room temperature. To remove excess antibodies, four additional wash-steps were performed in PBS/EDTA/BSA before incubation with fluorescein-isothiocyanate (FITC) conjugated goat anti-mouse IgG (Dako, Carpentaria, Calif., U.S.A.), diluted 1:80 in PBS, for 30 min. Platelets were washed again three times and resuspended in 100 μl PBS/EDTA/BSA. Fluorescence intensity was measured by FACSscan (Becton Dickinson, San Jose, Calif., U.S.A.).

Monoclonal antibodies. The expression of the platelet membrane glycoproteins was studied with the following MoAbs: CLB-thromb/1 (C17; CLB, Amsterdam, the Netherlands) and VPL2 (kindly provided by Prof. W. Knapp, Vienna, Austria) against GPIb–IIIa; MB45 and MB15, from our own laboratory, directed against the elastase-sensitive part of the GPIb–IIIa chain; MM2/174 (kindly provided by Dr D. Wilkinson, Victoria, Australia) and PHN103 (kindly provided by Dr Y. Bai, Beijing, China) directed against the elastase-resistant part of GPIbα chain; GII2 (kindly provided by Dr S. Santoso, Giessen, Germany), which recognizes a cryptic epitope on the β chain of GPIb; FMC25 (kindly provided by Dr H. Zola, Adelaide, Australia), BL-H6 (kindly provided by Prof. H. Fiebig, Leipzig, Germany) and GR-P (kindly provided by Dr E. Garrido, Granada, Spain) against GP Ib/IIIa; SW16 against GPIV; CLB-thromb/4 (10G11) against GPIIa–IIIa and ESIVC7 against GPIV. The last three MoAbs were from our own laboratory.

MoAb IgG1a recognizing plant allergens and IgG2a against TNP antigen were used as control antibodies.
Isolation and amplification of genomic DNA. Genomic DNA was isolated from mononuclear leucocytes by a conventional method (Ciulla et al. 1988).

The oligonucleotide primers used in the polymerase chain reaction (PCR) for amplifying the entire coding region of GPIX in a 712 bp product were designed from the published sequences of the GPIX gene (EMBL data bank, Heidelberg, Germany): primer 1 was a sense primer extended with a Hind III site at its 5’ end; primer 2, antisense, was extended with an Eco RI site at its 5’ end (Table I).

PCR was performed with 1 μg genomic DNA in a final volume of 50 μl. The reaction mixture contained 40 pmol of each primer, 0·2 μM of each dNTP (dATP, dCTP, dGTP, dTTP) from Promega, Madison, Wis., U.S.A.), 1·0 mM MgCl2 and 2 U of Taq DNA Polymerase (Promega) in the appropriate buffer. 35 amplification cycles were performed in a Thermal cycler (Perkin Elmer Cetus, model 480, Norwalk, Ct., U.S.A.) with denaturation at 95°C for 60 s, annealing at 55°C for 90 s and extension at 72°C for 150 s. The reaction was followed by a final 5 min incubation at 72°C to allow completion of strand synthesis. An aliquot of each PCR sample was separated by electrophoresis on 2% agarose gel, and stained with ethidium bromide, for the qualitative analysis of amplified PCR products.

Amplified DNA was subcloned into the vector pGEM-T (Promega) without previous purification and transformed into JM109 high-efficiency competent cells (Promega). Recombinants were selected by blue–white colour screening on indicator plates.

Nucleotide sequence analysis was performed by the dideoxy chain termination method using Sequenase 2.0 according to the manufacturer’s recommendations (US Biochemical Corp., Cleveland, Ohio, U.S.A.) and the appropriate primers.

Allele-specific oligonucleotide hybridization to amplified genomic DNA. Amplified genomic DNA was hybridized with 15-base allele-specific oligonucleotides listed in Table I: probe A corresponds to the published GPIX gene sequence (Hickey & Roth et al. 1993); probe B, differing from probe A only in the central base, a C instead of a T; was specific for the observed sequence of the patient’s GPIX gene.

Oligonucleotides were end-labelled using [γ-32P]ATP and T4 polynucleotide kinase (Boehringer Mannheim, Germany).

After DNA amplification, 10 μl of unpurified PCR products were diluted with 100 μl water and denatured for 3 min at 95°C. 110 μl of 10-times concentrated (10×) standard sodium citrate (SSC) (1× SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7·0) were added to the denatured samples. From each sample, two 100 μl mixtures were applied to a nylon membrane (Nylon Transfer Membrane, Amersham International, U.K.) using a slot-blot apparatus (Hybri-slot Manifold, BRL, Gaithersbourg, Md., U.S.A.) and vacuum suction. After a 10 min fixation in 0·2 M NaOH and one wash-step in 3× SSC, the membrane was prehybridized in 5× Denhardt’s solution, 5× SSC, 5 μg EDTA, 10 μg N2HPO4 at 44°C for 1 h. The filter was cut into two strips that were hybridized to either probe A or probe B by addition of 100 ng probe to prehybridization solution and incubation at 44°C for one additional hour.

Membranes were washed twice with 3× SSC at 44°C for 15 min and then twice more for 15 min at 45°C.

The hybridizations were visualized by exposing filters for 24 h to X-ray film at −70°C with an intensifier screen.

RESULTS

Flow cytometry studies

A panel of MoAbs directed against the main platelet membrane glycoproteins was used to evaluate their expression on the surface of platelets from our BSS patient and from a healthy unrelated donor. Flow cytometry results are shown in Table II and Fig 1. The percentage of labelled platelets from the patient and the donor was similar when MoAbs against GPIb–IIa were used. However, the mean fluorescence intensity in the patient was higher than normal because of his abnormally large platelets. Similar results were obtained when platelets were analysed for their GPIa–IIa and GPIV expression. The reactions with MoAbs recognizing different GPIbα epitopes showed that the expression of this glycoprotein was reduced on the patient’s platelets. A less pronounced reduction of reactivity was observed with SW16, a MoAb against GPVI. Only the platelets of the propositus reacted weakly with Gi22 that recognizes a cryptic epitope on GPIbβ. Finally, three MoAbs recognizing different epitopes on GPIX failed to react with the patient’s platelets.

Platelet glycoprotein expression was also investigated in

Fig 1. Flow cytometric analysis of surface glycoprotein expression of normal (upper panels) and BSS platelets (lower panels). Results are expressed as histograms of cell number (linear scale) on the ordinate versus fluorescence intensity (log scale) on the abscissa. Platelets were incubated with a MoAb specific for GPIIb–IIIa (VIPL), GPIIb (PHN103), GPIX (BL-H6), GPV (SW16) and with an irrelevant antibody (negative control). Bound antibodies were detected by FITC-anti-mouse IgG and analysed with a FACScan flow cytometer. Results obtained with VIPL, PHN103 and BL-H6 are representative of those obtained with all the other MoAbs specific for GPIIb–IIIa, GPIIb and GPIX, respectively.
one daughter of the patient. The expression of GPIIb–IIIa was increased with respect to control due to the presence of a detectable population of giant platelets (Fig 2); normal binding to GPIb and GPV was observed whereas the reaction with an anti-GPIX MoAb was little reduced.

Genetic characterization

In order to define the molecular basis of the GPIX deficiency, the gene encoding for the patient’s GPIX was analysed. The GPIX gene includes three exons and two introns, with the entire coding region contained within the third exon (Hickey & Roth, 1993). Therefore we performed molecular studies with genomic DNA isolated from patient and donor mononuclear leucocytes. Amplified PCR products from the patient and a donor were analysed on a 2% agarose gel. The mobility pattern was normal. After subcloning, nucleotide sequence analysis of amplified DNA was performed. The nucleotide T, normally present at position 1856 (according to sequence listed in EMBL data bank), was changed to a C in all nine clones obtained from the patient. This nucleotide difference predicts a substitution of a phenylalanine by a serine at amino-acid position 55 of the mature GPIX (Fig 3). Therefore the mutated amino acid is located in the unique GPIX leucine-rich repeat.

![Fig 2. Flow cytometry analysis of the membrane glycoproteins of platelets from the propositus’ daughter and from a normal donor. On the vertical axis the investigated GPs and their relative MoAbs are indicated.](image)

![Fig 3. Sequence analysis of amplified GPIX gene from genomic DNA derived from the BSS patient. The normal T at position 1856 (numbering according to sequence listed in EMBL bank) is changed to a C in the patient, predicting a phenylalanine to serine substitution at position 55 of the mature glycoprotein.](image)

**Table II.** Expression of the main platelet membrane glycoproteins in flow cytometric studies.

<table>
<thead>
<tr>
<th>MoAb</th>
<th>GP</th>
<th>Donor Gated (%)</th>
<th>Donor MFI</th>
<th>Patient Gated (%)</th>
<th>Patient MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>C17</td>
<td>IIb–IIIa</td>
<td>98.8</td>
<td>306</td>
<td>81.7</td>
<td>498</td>
</tr>
<tr>
<td>VIPL</td>
<td>IIb–IIIa</td>
<td>99.1</td>
<td>233</td>
<td>91.9</td>
<td>949</td>
</tr>
<tr>
<td>10G11</td>
<td>IIa–IIIa</td>
<td>82.6</td>
<td>23.9</td>
<td>94.3</td>
<td>75</td>
</tr>
<tr>
<td>ESIVC7</td>
<td>IV</td>
<td>98.4</td>
<td>89</td>
<td>94.0</td>
<td>347</td>
</tr>
<tr>
<td>MB45</td>
<td>Ibα es</td>
<td>93.6</td>
<td>413</td>
<td>41.5</td>
<td>34</td>
</tr>
<tr>
<td>MB15</td>
<td>Ibα es</td>
<td>96.7</td>
<td>551</td>
<td>46.0</td>
<td>32</td>
</tr>
<tr>
<td>PHN103</td>
<td>Ibα er</td>
<td>95.5</td>
<td>658</td>
<td>86.6</td>
<td>50</td>
</tr>
<tr>
<td>MM2/174</td>
<td>Ibα er</td>
<td>98.6</td>
<td>107</td>
<td>53.2</td>
<td>36</td>
</tr>
<tr>
<td>Gl22</td>
<td>Ibβ</td>
<td>1.0</td>
<td>ne</td>
<td>12.9</td>
<td>162</td>
</tr>
<tr>
<td>FMIC25</td>
<td>IX</td>
<td>99.0</td>
<td>135</td>
<td>15.7</td>
<td>37</td>
</tr>
<tr>
<td>BL-H6</td>
<td>IX</td>
<td>99.3</td>
<td>143</td>
<td>11.6</td>
<td>62</td>
</tr>
<tr>
<td>GR-P</td>
<td>IX</td>
<td>98.0</td>
<td>141</td>
<td>13.4</td>
<td>74</td>
</tr>
<tr>
<td>SW16</td>
<td>V</td>
<td>98.4</td>
<td>99</td>
<td>73.8</td>
<td>49</td>
</tr>
</tbody>
</table>

MFI: mean fluorescence intensity; es: elastase sensitive; er: elastase resistant; ne: not evaluable.

No additional nucleotide substitutions were identified. The normal T at position 1856 was found in all eight clones obtained from the donor.

Allele-specific oligonucleotide hybridization
The detected single-pair substitution at position 1856 of the gene coding for the patient’s GPIX neither created nor abolished the nucleotide sequence recognized by any of the known restriction enzymes. Therefore allele-specific hybridization was used to determine the distribution of the missense mutation among the available family members (a son, a daughter and a grandson of the propositus).

Probe B, which was specific for the mutated C at position 1856, hybridized with amplified DNA from the patient and both children (Fig 4). Probe A, recognizing the normal GPIX gene sequence, hybridized with DNA obtained from two normal donors, both children and the grandson of the propositus. Probe A did not hybridize with genomic DNA of the patient (Fig 4).

These results confirmed the homozygous presence of the T1856C mutation in the GPIX gene in our BSS patient. The nucleotide difference was transmitted to both unaffected studied children (Fig 5), whilst the grandson was shown not to be a carrier of the described molecular defect.

DISCUSSION
Several mutations affecting the α subunit of GPIb have been described in BSS patients (Ware et al, 1990, 1993; Miller et al, 1992; Simsek et al, 1994a, b; De La Salle et al, 1995; Li et al, 1995; Furihata et al, 1994; Kunishima et al, 1994; Lanza et al, 1993; Margaglione et al, 1995; Kanaji et al, 1995; Yamamoto et al, 1995), some of them located within its LRG motifs. A specific amino-acid substitution affecting the highly conserved leucine-57 residue in the α subunit of GPIb, detected in four BSS family members, was the first example of a molecular defect occurring in a leucine-rich repeat of a platelet membrane glycoproteins (Miller et al, 1992). In that case, the GPIbα defect caused an important bleeding tendency transmitted in an autosomal dominant manner. Furthermore, another point mutation in an LRG repeat of the same molecule resulted in a variant form of BSS (Ware et al, 1993). The alanine-156 to valine substitution was responsible, in this case, for a defective binding of von Willebrand factor.

However, single mutations occurring within the von Willebrand factor binding domain of GPIbα may result in increased binding of von Willebrand factor. Indeed, the reported glycine-233 to valine and methionine-239 to valine substitution in the GPIbα gene were responsible for platelet-type von Willebrand disease (Miller et al, 1991; Russel & Roth, 1993; Takahashi et al, 1995).

Double heterozygosity for mutations in the platelet GPIX gene has been identified in three affected siblings from one

family with BSS (Wright et al., 1991). Two different heterozygous missense mutations leading to a conversion of an aspartic acid-21 to glycine and an asparagine-45 residue to serine were the molecular basis for the congenital bleeding disorder in that family. Both amino acid substitutions affected conserved residues within or flanking the single GPIX LRG motif. The same asparagine-45 to serine mutation has been described in homozygous form in another BSS patient (Clemetson et al., 1994), suggesting a major tendency to mutation of the GPIX 45 codon. Moreover, a homozygous nonsense mutation at codon 126 of the GPIX gene was recently shown to be associated with BSS (Noda et al., 1995a) as well as a missense mutation affecting the codon 73 of the gene (Noda et al., 1995b).

In this study we report additional evidence that a molecular defect in the GPIX gene can result in BSS. We identified a point mutation within the GPIX coding region for which the patient was homozygous: a T to C mutation which predicts a phenylalanine to serine substitution at position 55 in the mature glycoprotein, within the unique LRG repeat of GPIX. In immunofluorescence studies a very low or absent expression of GPIX was found, associated with a decreased GPIbα and GPV expression. Additionally, an increased binding to a MoAb recognizing a cryptic epitope on GPIbβ was detected on the patient’s platelets: this is compatible with the disruption of the GPIb–IX–V structure and the exposure of an usually inaccessible epitope on GPIbβ. By allele-specific oligonucleotide hybridization we recognized no normal alleles in the patient, who has probably inherited the molecular defect from both parents. A carrier state for the phenylalanine-55 to serine substitution was shown in both children studied. In one of them, reduced GPIX expression on the platelet surface was also detected.

Because of its LRG domain location, we can speculate that the phenylalanine-55 to serine mutation, probably resulting in an alteration of the structure of GPIX, may prevent the normal assembly of the GPIb–IX–V complex with, as a consequence, decreased GPIb expression on the platelet surface in addition to the undetectability of GPIX. A similar finding was described for the GPIX defect reported by Clemetson et al. (1994): substitution of asparagine-45 to serine in the LRG domain was responsible not only for a markedly reduced GPIX expression, but also for a significantly impaired expression of GPIbα, Ibβ and V. The authors postulated that GPIX is probably important for an efficient expression of the total complex, but not essential for the complexation of GPIbα and Ibβ with GPV. The need for GPIX for a correct assembly and membrane expression of the GPIb–IX–V complex was confirmed in another recently described GPIX defect (Noda et al., 1995a). A nonsense mutation had occurred at nucleotide 126 of the GPIX coding region, probably resulting in an abnormal molecule lacking the C-terminal side including the transmembrane domain. This mutation led to a severely reduced expression of GPIb and GPV. All these results are in agreement with previous reports, namely that for an efficient expression of GPIb–IX in transfected cells, all three subunits Ibo, Ibβ and IX are required (Clemetson et al., 1982; Arai et al., 1994; Lopez et al., 1992, 1995).

In our patient the amount of GPV complement was only slightly reduced compared to normal levels, which is in accordance with the finding that free unassociated GPV is also present on platelets. Unlike GPIbα, GPIbβ and GPIX, in the above-mentioned transfection studies the GPV subunit, that is non-covalently associated with GPIb–IX (Modderman et al., 1992), has been found not to be essential for the expression of the GPIb–IX complex in the platelet plasma membrane (Simske et al., 1994b; Arai et al., 1994; Lopez et al., 1992, 1995).

In conclusion, we have described homozygosity for a point mutation in the unique LRG motif of the GPIX gene of a BSS patient. This nucleotide substitution caused a lack of expression of GPIX on the platelet membrane. It is likely that this mutation, in our case, interfered with the normal assembly of the GPIb–IX complex resulting in a BSS phenotype. However, more transfection studies are necessary to evaluate how the single components of GPIb–IX–V are involved in an efficient formation and expression of the complex. Further investigations are also required to study the role that LRG domains play in the interactions between all four complex subunits.

ACKNOWLEDGMENTS

We thank L. Porcelijn, E. Huiskes, P. M. Bleeker, B. Bossers, H. Bruinink, L. Buddelmeijer, I. Dammsa and K. van der Kolk for their technical support. We also thank Professor C. L. Baldini and Professor C. P. Engelfriet for their helpful and critical discussions.

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