The heptapeptide LSARLAF mediates platelet activation through phospholipase C γ 2 independently of glycoprotein IIb-IIIa

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The seven-amino-acid peptide LSARLAF has been reported to activate platelets via the integrin GPIIb-IIIa (glycoprotein IIb-IIIa). Activation by LSARLAF is reinforced by release of ADP and thromboxanes, but the initiating event in the signalling cascade is not known. In the present study, we demonstrate that LSARLAF stimulates Src kinase-dependent tyrosine phosphorylation of many of the proteins in the GPIIb-IIIa cascade, including the tyrosine kinase Syk, the adapter SLP-76 (SH2-containing leucocyte phosphoprotein of 76 kDa) and PLC γ 2 (phospholipase C γ 2). A critical role for PLC γ 2 in signalling by LSARLAF was demonstrated by abolition of aggregation in PLC γ 2^{−/−} murine platelets to low concentrations of the peptide, although a partial recovery was seen with higher concentrations. In sharp contrast with the GPIIb-IIIa-regulated signalling cascade, aggregation was inhibited in murine platelets deficient in the adapter LAT (linker for activation of T-cells) and the Fc receptor γ -chain. Aggregation was also partially inhibited by the cholesterol-lowering

reagent, β -methyl-cyclodextrin, at concentrations that disrupt membrane rafts, but do not interfere with signalling by GPIIb-IIIa. Furthermore, LSARLAF also stimulated tyrosine phosphorylation in GPIIb-deficient murine platelets, confirming that the integrin is not critical for activation of intracellular signalling pathways. LSARLAF also stimulated Ca²⁺ elevation in RBL-2H3 cells, which lack the platelet glycoproteins GPIIb, GPVI and GPIb. These results demonstrate that LSARLAF activates platelets through a PLC γ 2-dependent pathway that lies downstream of Src kinases and which is partially dependent on the Fc receptor γ -chain, LAT and lipid rafts. The mechanism of cell activation by LSARLAF remains to be established, although the present results indicate that more than one surface glycoprotein may mediate this response.

Key words: LSARLAF, Fc receptor γ -chain, glycoprotein VI, integrin α IIb β 3, phospholipase C γ 2, platelet.

INTRODUCTION

The heptapeptide LSARLAF was designed by a complementary peptide-modelling approach [1] to bind to the platelet fibrinogen receptor, the integrin GPIIb-IIIa (glycoprotein IIb-IIIa). The direct binding of LSARLAF to GPIIb-IIIa was confirmed by binding assays *in vitro* [1]. LSARLAF promotes strong platelet aggregation and secretion responses, which are reinforced by release of ADP and thromboxanes [2,3]. Antagonists of the RGD-binding site of GPIIb-IIIa do not block LSARLAF-induced secretion, indicating that LSARLAF can cause platelet activation without the need for GPIIb-IIIa ligation by fibrinogen or platelet aggregation [1]. The ability of LSARLAF to stimulate secretion was abolished in platelets from a patient with Type I Glanzmann's thrombocytopenia and from a patient with a deficiency in GPVI, suggesting a critical role for the two glycoproteins in activation by the heptapeptide [4,5]. However, the molecular basis of platelet activation by LSARLAF remains to be established.

The signalling pathways that are regulated by GPIIb-IIIa and GPVI have been the subject of extensive research. One of the earliest events in outside-in signalling by GPIIb-IIIa is the phosphorylation and activation of the tyrosine kinase Syk by Src family kinases [6–8]. Signalling by GPIIb-IIIa is accompanied by tyrosine phosphorylation of the β 3 integrin subunit [9] and the adaptor SLP-76 (SH2-containing leucocyte phosphoprotein of 76 kDa), which is constitutively associated with the adap-

tors ADAP [adhesion- and degranulation-promoting adapter protein, previously known as SLAP-130 (SLP-76-associated phosphoprotein of 130 kDa) and Fyb] and Gads (Grb2-related adapter downstream of Shc) [10–13]. The summation of these adaptor interactions leads to activation of PI3K (phosphoinositide 3-kinase) and PLC γ 2 (phospholipase C γ 2) and remodelling of the cytoskeleton [9,14–16].

GPIIb-IIIa signalling shares many similarities with signalling by the collagen receptor complex, the GPVI-FcR γ -chain complex [17,18] and by the receptor for von Willebrand factor, GPIb-IX-V. This includes the sequential activation of Src and Syk tyrosine kinases and subsequent tyrosine phosphorylation of SLP-76 and activation of PI3Ks and PLC γ 2 [19–25]. However, in contrast with signalling by GPIIb-IIIa, platelet activation by GPVI is critically dependent on the FcR γ -chain [17] and is partially dependent on the transmembrane adapter LAT (linker for activation of T-cells) [26], both of which are concentrated in specialized membrane microdomains known as lipid rafts or GEMS (glycolipid-enriched membrane microdomains) [27]. GPIIb-IIIa is excluded from GEMS and does not require either of these proteins for activation of PLC γ 2 in platelets [16,27]. There are contrasting reports on the role of GEMS and the FcR γ -chain in signalling by GPIb-IX-V [25,28,29].

The aim of the present study was to delineate the molecular basis of platelet activation by LSARLAF by comparison of the signalling cascades used by the peptide and those activated by

Abbreviations used: CRP, collagen-related peptide; FcR γ -chain, Fc receptor γ -chain; GEMS, glycolipid-enriched membrane microdomains; GPIb, glycoprotein Ib; GPIIb-IIIa, glycoprotein IIb-IIIa; GPVI, glycoprotein VI; LAT, linker for activation of T-cells; PI3K, phosphoinositide 3-kinase; PA, phosphatidic acid; PLC γ 2, phospholipase C γ 2; PP1, 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo-D-3,4-pyrimidine; PRP, platelet-rich plasma; SLP-76, SH2-containing leucocyte phosphoprotein of 76 kDa.

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GPIIb-IIIa and GPVI. We show that LSARLAF activates platelets via a Src kinase-dependent pathway that leads to activation of PLC γ 2. This pathway is partially dependent on LAT and the FcR γ -chain. Interestingly, however, LSARLAF is able to induce activation of platelets and RBL-2H3 cells in the absence of GPIIb-IIIa, GPVI and GPIb-IX-V, demonstrating that none of these receptors are critical for activation. Part of this work has been previously presented in abstract form [5].

EXPERIMENTAL

Antibodies and reagents

Anti-phosphotyrosine monoclonal antibody 4G10 and anti-LAT polyclonal antibody were purchased from Upstate Biotechnology (TCS Biologicals, Botolph Claydon, Buckingham, Bucks., U.K.). The anti-PLC γ 2 and anti-Syk polyclonal antibodies were kindly supplied by Dr Mike Tomlinson (DNAX, Palo Alto, CA, U.S.A.). The anti-SLP-76 polyclonal antibody was a kind gift from Dr Gary Koretzky (University of Pennsylvania, Philadelphia, PA, U.S.A.). The anti- β 3 polyclonal antibody was a kind gift from Dr Sanford Shattil (The Scripps Institute, La Jolla, CA, U.S.A.). The PLC inhibitor U73122 was purchased from Calbiochem (Nottingham, U.K.). LSARLAF and the scrambled control peptide FRALASL were purchased from Tana Laboratories (Houston, TX, U.S.A) and Calbiochem. LSARLAF was also made as described previously [30]. All other reagents were purchased from Sigma (Poole, Dorset, U.K.) or obtained from sources described previously [24,26,27].

Animals

FcR γ -chain deficient mice [31] were bred as homozygotes using C57/BL6 wild-type mice as controls. PLC γ 2-, LAT- and GPIIb-deficient mice were generated as described previously [32–34], and bred as heterozygotes using litter-matched wild-type mice as controls. All animals were maintained using housing and husbandry in accordance with local and national legal regulations.

Preparation of human platelets

Blood was taken by forearm venepuncture from healthy drug-free volunteers on the day of the experiment and diluted in 1:10 (v/v) sterile sodium citrate. PRP (platelet-rich plasma) was obtained by centrifugation of the blood at 200 *g* for 20 min. Platelets were isolated from PRP by centrifugation at 1000 *g* for 10 min in the presence of 0.1 μ g/ml prostacyclin. The platelet pellet was resuspended in modified Hepes-Tyrode's buffer [134 mM NaCl/0.34 mM Na₂HPO₄/2.9 mM KCl/12 mM NaHCO₃/20 mM Hepes/5 mM glucose/1 mM MgCl₂ (pH 7.3)]. The platelets were centrifuged at 1000 *g* for 10 min in the presence of 0.1 μ g/ml prostacyclin and 1:9 (v/v) acid citrate dextrose (120 mM sodium citrate/110 mM glucose/80 mM citric acid) and resuspended at a concentration of 5×10^8 cells/ml in Hepes-Tyrode's buffer.

Preparation of mouse platelets

Blood was taken by cardiac puncture from a mouse killed by CO₂ asphyxiation on the day of the experiment and diluted to 1:10 (v/v) in acid citrate dextrose. Blood was diluted 1:6 (v/v) in Hepes-Tyrode's and centrifuged at 200 *g* to obtain PRP. PRP was centrifuged in the presence of 0.1 μ g/ml prostacyclin at 1000 *g*. The platelet pellet was resuspended at a concentration of 5×10^8 cells/ml in Hepes-Tyrode's buffer.

Platelet stimulation and aggregation

Human platelets were used at a concentration of 2×10^8 cells/ml for aggregation studies or 5×10^8 cells/ml for protein studies. Mouse platelets were used at a concentration of 2×10^8 /ml. For all protein studies, lotrafiban (10 μ M), indomethacin (10 μ M) and apyrase (2 units/ml) were included in the resuspension buffer, unless otherwise stated. Stimulation of platelets was performed in a PAP-4 aggregometer (Bio/Data Corps, Horsham, PA, U.S.A.) with continuous stirring at 1200 rev./min at 37 °C for the times indicated. Aggregation of platelets was monitored by measuring changes in light transmission in the absence of lotrafiban, indomethacin and apyrase. Platelets were preincubated with U73122 (20 μ M) or PP1 (4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo-D-3,4-pyrimidine; 20 μ M) for 10 min prior to stimulation by LSARLAF where indicated. Washed platelets were depleted of cholesterol using β -methyl-cyclodextrin as described previously [27].

Immunoprecipitation and immunoblotting

Platelets were lysed with an equal volume of $2 \times$ lysis buffer [2 % Nonidet P40/300 mM NaCl/20 mM Tris/10 mM EDTA/2 mM Na₃VO₄/200 μ g/ml 4-(2-aminoethyl)-benzenesulphonylfluoride hydrochloride/10 μ g/ml leupeptin, 10 μ g/ml aprotinin/1 μ g/ml pepstatin A, pH 7.4]. Insoluble cell debris was removed by centrifugation for 5 min at 13 400 *g* at 4 °C and cell lysates were precleared using Protein A-Sepharose. Platelet lysates were incubated with the indicated primary antibodies, and the resulting protein complexes and immunoprecipitates were resolved by SDS/PAGE and transferred to PVDF membranes. Immunoblotting was performed as described previously [16], with detection by enhanced chemiluminescence (ECL[®], Amersham Biosciences, Little Chalfont, Bucks., U.K.).

Measurement of PA (phosphatidic acid) production

Platelets were resuspended in Hepes-Tyrode's without phosphate and incubated with [³²P]P_i (0.5 mCi/ml) for 1 h at 37 °C. Platelets were washed and used for experimentation as described above. The reaction was stopped by addition of 1 volume of chloroform/methanol/HCl (100:200:1, by vol.), phospholipids extracted and [³²P]PA separated by TLC and analysed by autoradiography and densitometry.

Tissue culture

RBL-2H3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 100 units/ml penicillin, 100 μ g/ml streptomycin and 10 % heat-inactivated foetal bovine serum in 5 % CO₂/95 % air in a humidified incubator. Cells were maintained at exponential phase of growth. Adherent cells were detached by incubation with trypsin/EDTA for 5 min at 37 °C before washing.

Ca²⁺ fluorimetry

RBL-2H3 cells were resuspended in RPMI 1640 (Life Technologies) with 25 mM Hepes containing 1 mg/ml BSA. Cells were loaded with the Ca²⁺ reporter dye fura 2 by incubation with 3 μ M fura 2 acetoxymethyl ester for 1 h at 37 °C. Cells were stimulated with slow stirring in a spectrofluorimeter at 37 °C with excitation wavelengths of 330 nm and 380 nm. Emission at 510 nm was measured, and the ratio of fluorescence analysed

using FWinlab software. Ratiometric analysis was converted into Ca^{2+} concentration.

Analysis of data

Experiments were carried out on at least three occasions and are shown as representative data from one experiment. Where applicable, results are expressed as the means $\pm$ S.E.M.

RESULTS

LSARLAF causes platelet aggregation via activation of Src kinases and PLC

The ability of LSARLAF to activate platelets was investigated by monitoring aggregation. A threshold concentration of LSARLAF (250 μM) stimulated shape change, with higher concentrations (0.5–1.4 mM) inducing partial to maximal aggregation (Figure 1, i and ii). As previously reported [1], the scrambled control peptide FRALASL had no effect on washed platelet suspensions (results not shown). Aggregation induced by LSARLAF (750 μM) was blocked by lotrafiban, an antagonist of the RGD-binding site on GPIIb-IIIa, demonstrating that it was mediated by activation of the integrin. However, a residual lotrafiban-insensitive increase in light transmission remained, which was due to passive cross-linking of platelets, as reported previously [5,35]. The response to LSARLAF was inhibited in the presence of the Src kinase inhibitor PP1 and the pan-isoform PLC inhibitor U73122 (Figure 1, iii) to a similar extent to that seen with lotrafiban. These results demonstrate that LSARLAF stimulates aggregation through a pathway that is dependent on Src kinases and PLC.

LSARLAF stimulates protein tyrosine phosphorylation and activation of PLC γ 2

To further investigate the Src kinase-dependent component of the response to LSARLAF, we monitored tyrosine phosphorylation in whole-cell lysates using the pan anti-phosphotyrosine monoclonal antibody, 4G10. Aggregation was blocked with lotrafiban, to prevent outside-in signals arising from the cross-linking of GPIIb-IIIa by fibrinogen. Signals by released ADP and thromboxanes were blocked with apyrase and indomethacin respectively. LSARLAF stimulated a marked increase in tyrosine phosphorylation, with prominent bands at molecular masses of 26, 30, 70 and 150 kDa (indicated by arrows), as well as a number of minor bands (Figure 2, i). Immunoprecipitation studies using specific antibodies identified Syk, SLP-76 and PLC γ 2 among the tyrosine-phosphorylated proteins. In comparison, LAT showed only a small increase in tyrosine phosphorylation (Figure 2, ii–v). Similarly, the FcR γ -chain found in Syk immunoprecipitates showed only a small increase in tyrosine phosphorylation. The FcR γ -chain in the Syk immunoprecipitates was identified by Western blotting with an anti-FcR γ -chain antibody (Figure 2, ii). When the platelets were stimulated in the absence of lotrafiban, the platelets underwent aggregation and tyrosine phosphorylation of the integrin β 3 subunit was observed (Figure 2, vi).

The ability of LSARLAF to stimulate formation of [^{32}P]PA was measured to address the functional relevance of tyrosine phosphorylation of PLC γ 2. PA is an indirect marker of activation of PLC and is formed by metabolism of 1,2-diacylglycerol. LSARLAF stimulated a similar increase in formation of PA to that induced by the GPVI-specific agonist convulxin (Figure 3, i). The ability of LSARLAF to activate PLC was confirmed by measurement of formation of inositol phosphates in platelets

Figure 1 LSARLAF-induced platelet aggregation is blocked by inhibitors of Src kinases and PLC

(i) Washed human platelets were stimulated with the indicated concentrations of LSARLAF (LSA) in an aggregometer. The addition of the agonist is indicated with an arrow. The change in D , indicative of the percentage of aggregation, was monitored. (ii) The final extent of aggregation of human platelets was plotted against the concentration of LSARLAF and is expressed as the means $\pm$ S.E.M. (iii) Washed human platelets were incubated with 10 μM lotrafiban, 20 μM PP1, 20 μM U73122 or 0.1% DMSO (control). Platelets were stimulated with 750 μM LSARLAF in an aggregometer and the D , indicative of the percentage of aggregation, was monitored. Addition of agonist is indicated with an arrow. One experiment is shown that is representative of three.

metabolically labelled with [^3H]inositol (results not shown). Significantly, the formation of PA by LSARLAF was not inhibited by lotrafiban, demonstrating that it is independent of engagement of GPIIb-IIIa by fibrinogen (Figure 3, i). These results indicate that stimulation of tyrosine phosphorylation of PLC γ 2 by LSARLAF leads to activation of PLC γ 2.

LSARLAF activates murine platelets via PLC γ 2

The ability of LSARLAF to activate murine platelets was investigated as a basis on which to perform studies on mutant mice. Murine platelets underwent shape change and aggregation to LSARLAF over the same concentration range as seen for human platelets (for example, see Figure 3, ii). At a concentration of 750 μM , LSARLAF induced a slightly greater degree of

Figure 2 LSARLAF causes tyrosine phosphorylation of signalling proteins including PLC γ 2

Washed human platelets were stimulated in an aggregometer for 60 s in the presence of 10 μ M indomethacin and 2 units/ml apyrase. In samples (i–v), 10 μ M Iotrafiban was also included. The reactions were stopped by addition of 2 $\times$ reducing Laemmli buffer (i) or 2 $\times$ immunoprecipitation lysis buffer (ii–vi). Whole cell lysate (i) or immunoprecipitations of the indicated proteins were separated by SDS/PAGE (10% gel) and immunoblotted with anti-phosphotyrosine antibody (α -p-tyr). Immunoprecipitates (ii–vi) were stripped and reprobed for the indicated immunoprecipitated proteins. One experiment is shown for each, which is representative of at least three separate experiments. α , anti-; IP, immunoprecipitation; WB, Western blot.

aggregation than in human platelets (human, $56.75 \pm 6.1\%$; mouse, $81.6 \pm 2.9\%$).

PLC γ 2-deficient murine platelets were used to assess the role of PLC γ 2 in platelet activation by LSARLAF. Platelet aggregation induced by LSARLAF (500 μ M) was markedly inhibited, but not abolished, in PLC γ 2-deficient cells (Figure 3, ii). The response to a higher concentration of LSARLAF (1 mM) was only partially (40%) reduced in the PLC γ 2-deficient cells (Figure 3, ii), whereas the PLC inhibitor U73122 reduced the response by more than 80% (results not shown), suggesting a role for PLC γ 1 and possibly other PLC isoforms in mediating activation. The residual response in the presence of U73122 may represent passive agglutination as discussed above.

These studies were extended to platelets deficient in the adapter LAT and FcR γ -chain. In the absence of FcR γ -chain, aggregation to a submaximal dose of LSARLAF (500 μ M) was abolished, although shape change was observed (Figure 4, i), demonstrating a residual level of activation. In comparison, aggregation in response to LSARLAF (500 μ M) was markedly inhibited, but not abolished, in the absence of LAT (Figure 4, i). Interestingly, in both sets of platelets, the response to a higher concentration of LSARLAF (1 mM) was only marginally inhibited relative to wild-type controls (Figure 4, i). This demonstrates that LAT and FcR γ -chain are involved in platelet activation by LSARLAF, but that neither are essential.

The loss of response to a submaximal concentration of LSARLAF (500 μ M) in LAT- and FcR γ -chain-deficient platelets was accompanied by a reduction in tyrosine phosphorylation of

PLC γ 2 (Figure 4, ii). Recovery of phosphorylation was seen in response to a higher concentration of LSARLAF (1 mM), although this was still reduced relative to the controls (Figure 4, ii).

The adapter LAT is almost exclusively localized in cholesterol-rich membrane microdomains, known as lipid rafts. We investigated the effect of the cholesterol-depleting agent β -methylcyclodextrin on LSARLAF-induced platelet aggregation at a concentration which has been shown to block activation by GPVI, but not by the phorbol ester PMA. β -Methylcyclodextrin inhibited the aggregation response to CRP (collagen-related peptide) and delayed the response to LSARLAF, but had no effect on the response to PMA (Figure 4, iii).

These observations demonstrate that LSARLAF stimulates platelet activation through a Src kinase-dependent pathway that leads to activation of PLC γ 2 and is partially dependent on FcR γ -chain, LAT and GEMS (glycolipid-enriched membrane microdomains).

LSARLAF induces tyrosine phosphorylation of PLC γ 2 and Ca $^{2+}$ mobilization independently of GPIIb-IIIa

The above results demonstrate a role for LAT and the FcR γ -chain in the activation of platelets by LSARLAF, two proteins that do not form part of the GPIIb-IIIa-signalling cascade [16]. In view of this, we performed additional studies to investigate the role of the integrin GPIIb-IIIa in platelet activation by LSARLAF.

Figure 3 LSARLAF-induced aggregation is reduced in PLCγ2 deficient murine platelets

(i) For analysis of PA levels, human platelets were labelled with [³²P]P_i, and stimulated with 750 μM LSARLAF (LSA), in the presence or absence of Iotafiban (Iot), or by 10 μg/ml convulxin (Cvx) for 5 min at 37 °C, and PA was extracted as described in the Experimental section. PA levels were detected by autoradiography of the TLC plate. (ii) Washed platelets from wild-type or mice deficient in PLCγ2 were stimulated with 500 μM or 1 mM LSARLAF in an aggregometer in the absence of Iotafiban, indomethacin and apyrase, and the *D*, indicative of the percentage aggregation, was monitored. One experiment is shown for each, which is representative of at least three separate experiments.

These experiments were performed in murine platelets with an engineered null mutation at the GPIIb locus, making them deficient in the GPIIb subunit of the integrin [34]. These mice do not express GPIIb [34].

PLCγ2 was immunoprecipitated from GPIIb-deficient murine platelet lysates activated with 750 μM LSARLAF. LSARLAF stimulated a similar increase in tyrosine phosphorylation of PLCγ2 in control and GPIIb-deficient platelets (Figure 4, iv), thereby demonstrating that the integrin is not essential for intracellular signalling by LSARLAF.

The results in the GPIIb- and FcR γ-chain-deficient mice demonstrate that LSARLAF is able to stimulate platelets in the absence of GPIIb-IIIa or the GPVI-FcR-γ-chain complex. To confirm the ability of LSARLAF to signal via a pathway that is independent of these two receptors, we measured the ability of the peptide to induce elevation of Ca²⁺ in a rat mast cell line, RBL-

2H3, which does not express either GPIIb-IIIa or GPVI (Figure 5). LSARLAF stimulated an increase in Ca²⁺ over the same concentration range as that which induced platelet activation. This is a specific effect of LSARLAF, as the scrambled peptide, FRALASL, did not alter basal fluorescence (results not shown).

DISCUSSION

In the present study we have investigated the molecular mechanism of LSARLAF-induced platelet activation. LSARLAF causes shape change and aggregation of human and murine platelets over a similar concentration range. LSARLAF induces GPIIb-IIIa activation through a pathway that is critically dependent on the activation of Src kinases and PLC, most notably the PLCγ2 isoform. In addition, activation by LSARLAF is partially dependent on the FcR γ-chain and LAT, although the requirement for either of these proteins is overcome at higher concentrations of the peptide. Several other proteins also undergo tyrosine phosphorylation in response to LSARLAF, including β3-integrin, Syk and SLP-76. Thus LSARLAF stimulates platelets through a tyrosine-kinase-dependent mechanism, which utilizes many of the proteins that are regulated by several of the major platelet glycoprotein receptors, including GPIIb-IIIa, GPIb and GPVI. Moreover, activation by LSARLAF is inhibited in the presence of the Src kinase inhibitor PP1, demonstrating that it is mediated by a specific signalling pathway rather than by, for example, non-specific permeabilization of the membrane.

It is presently unclear whether LSARLAF activates platelets through a single unidentified glycoprotein, through more than one surface receptor or through a non-specific mechanism. Accumulating evidence, however, argues in support of the latter two, rather than the first of these possibilities. The observation of a critical role for the FcR γ-chain and LAT in the activation of platelets by low, but not high, concentrations of LSARLAF demonstrates that activation by the seven-amino-acid peptide is not mediated solely through GPIIb-IIIa or GPVI. The integrin GPIIb-IIIa has been shown to stimulate platelets independently of these two proteins, whereas expression of GPVI at the plasma membrane requires the FcR γ-chain. Confirmation that LSARLAF does not require the integrin for activation was derived using GPIIb-deficient murine platelets. Moreover, LSARLAF was also shown to activate rat RBL-2H3 mast cells, which lack GPVI and GPIIb-IIIa. Thus neither GPIIb-IIIa nor GPVI function as the sole receptor for LSARLAF.

Nevertheless, additional support is required to confirm that either of these proteins serve as a functional receptor for the seven-amino-acid peptide, as phosphorylation of PLCγ2 by LSARLAF was not altered in GPIIb-deficient platelets, whereas the dose-response curve of Ca²⁺ mobilization by the peptide in RBL-2H3 cells was not altered by expression of GPVI (results not shown).

The platelet surface glycoprotein GPIb is a further potential receptor for LSARLAF as it signals through tyrosine phosphorylation of many of the proteins that are regulated by LSARLAF, including the FcR γ-chain, Syk and PLCγ2 [25,28]. The role of GPIb in signalling by LSARLAF, however, has not been addressed in the present study, as we do not have access to platelets which are deficient in the complex and also these platelets are unusually large thereby influencing the interpretation of the results.

It should also be taken into account, however, that we are unable to rule against the possibility that the effects of LSARLAF are mediated by a non-receptor mechanism. A possible non-receptor-based mechanism for platelet activation by LSARLAF could, for example, be mediated by an effect on surface charge at the

Figure 4 LSARLAF-induced aggregation is reduced in the absence of LAT, FcR γ -chain and GEMS, and PLC γ 2 tyrosine phosphorylation is maintained in GPIIb-deficient murine platelets

(i) Washed platelets from wild-type (WT) or FcR- γ -chain- or LAT-deficient ($-/-$) mice were stimulated with 500 μ M or 1 mM LSARLAF (LSA) in an aggregometer in the absence of lotrafiban, indomethacin and apyrase, and the D , indicative of the percentage aggregation, was monitored. (ii) Washed platelets from wild-type or FcR- γ -chain- or LAT-deficient ($-/-$) mice were stimulated in an aggregometer for 60 s in the presence of 10 μ M lotrafiban, 10 μ M indomethacin and 2 units/ml apyrase. The reactions were stopped by addition of $2 \times$ immunoprecipitation lysis buffer. Immunoprecipitations (IP) of PLC γ 2 were separated by SDS/PAGE (10%) and immunoblotted (WB) with anti-phosphotyrosine antibody (α -p-tyr) (upper panels). Immunoblots were stripped and reprobed for PLC γ 2 (lower panels). (iii) Washed human platelets treated with 22 mM β -methyl-cyclodextrin (β -M-CD) or Hepes-Tyrosine buffer as control during the washing procedure were stimulated in an aggregometer in the absence of lotrafiban, indomethacin and apyrase with the indicated concentrations of CRP, PMA, fibrinogen or LSARLAF. The D , indicative of the percentage aggregation, was monitored. (iv) Washed platelets from wild-type or GPIIb-deficient (IIb- $-/-$) mice were stimulated in an aggregometer for 60 s in the presence of 10 μ M lotrafiban, 10 μ M indomethacin and 2 units/ml apyrase. The reaction was stopped by addition of $2 \times$ immunoprecipitation lysis buffer. Immunoprecipitations of PLC γ 2 were separated by SDS/PAGE (10% gel) and immunoblotted with anti-phosphotyrosine antibody. Immunoprecipitates were stripped and reprobed for PLC γ 2 (lower panel). One experiment is shown, which is representative of at least three separate experiments.

Figure 5 LSARLAF causes Ca^{2+} mobilization in RBL-2H3 cells

RBL-2H3 cells loaded with the Ca^{2+} reporter dye fura-2 were stimulated with 250 μ M or 750 μ M LSARLAF (LSA) in a spectrofluorimeter with slow stirring. The ratio of fluorescence in response to excitation at 330 nm and 380 nm was converted to a Ca^{2+} concentration and plotted against time. One experiment is shown, which is representative of at least two separate experiments.

membrane, thereby causing glycoproteins to cluster and generate intracellular signals. If this is the case for LSARLAF, however, it is a sequence specific effect, as the scrambled peptide FRALASL does not induce platelet activation.

We have recently reported that the short peptide 4N-1, which is derived from the C-terminus of thrombospondin, is able to activate platelets through a pathway that is similar to that used by GPVI [36], but which also shares a number of characteristics with activation by LSARLAF. Although the sequence of 4N-1 is

distinct from that of LSARLAF, both peptides induce a similar pattern of tyrosine phosphorylation and stimulate significant FcR γ -chain-independent activation. It is possible that the two peptides stimulate a common receptor or induce activation through a shared non-receptor mechanism.

Rhodocytin (also known as aggregrin), a heterodimeric C-type lectin from the venom of the snake *Calloselasma rhodostoma*, has recently been shown to cause platelet activation through an uncharacterized receptor that mediates activation of Src family kinases, Syk and PLC γ 2 [37,38]. It is therefore of note that the signalling pathway activated by LSARLAF has a number of similarities to that used by rhodocytin, including an FcR- γ -chain-independent component. It is therefore possible that the rhodocytin receptor may play a role in LSARLAF-induced platelet activation.

In summary, the platelet agonist LSARLAF has been shown to activate platelets through a tyrosine-kinase-coupled pathway that involves Src family kinases and PLC γ 2. This pathway is also partially dependent on the FcR γ -chain, LAT and lipid rafts. The mechanism underlying this effect remains to be established, although it is not solely dependent on the platelet glycoproteins, GPVI and GPIIb-IIIa. We speculate that LSARLAF is an agonist at more than one receptor on the platelet surface, at least one of which signals through the FcR γ -chain. The latter may be the collagen receptor GPVI or possibly GPIb, but this requires supporting evidence. LSARLAF may help to identify novel tyrosine-kinase-linked receptors that cause calcium mobilization in platelets and in other cells.

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