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Crystallization of the OP-G2 Fab fragment: a fibrinogen mimic with specificity for the platelet glycoprotein IIb/IIIa.

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Abstract

The OP-G2 monoclonal antibody binds to the platelet integrin, gpIIb/IIIa, in a mode that mimics fibrinogen binding. The specificity of this antibody is mediated by the third complementarity-determining region (CDR3) loop of the immunoglobulin heavy chain which contains a sequence (RYD) related to the RGD recognition sequence of fibrinogen. The OP-G2 Fab fragment has been crystallized by vapor diffusion from solutions containing polyethylene glycol and imidazole malate (pH 5.6). The crystals belong to space group $P2_12_12$ with $a = 93.1$, $b = 83.8$ and $c = 53.7$ Å. One Fab molecule is present in the asymmetric unit. A complete data set to 2.0 Å resolution has been collected.

Introduction

Platelet aggregation is an important cellular interaction that occurs after external trauma to the vasculature. Stimulated platelets aggregate and this event initiates hemostasis. The membrane glycoprotein that mediates platelet aggregation is a non-covalently associated heterodimer, gpIIb/IIIa, which is a member of a superfamily of cell surface receptors called integrins (Hynes, 1987; Ruoslahti & Pierschbacher, 1987). Integrins mediate a number of diverse cell-adhesion phenomena through recognition of the sequence Arg-Gly-Asp (RGD) present in proteins such as fibrinogen, fibronectin and von Willebrand factor. The binding of gpIIb/IIIa to fibrinogen and von Willebrand factor causes platelets to aggregate and this binding results, in part, from recognition of the RGD sequence motif by the platelet integrin. Synthetic peptides corresponding to the RGD sequences in fibrinogen or von Willebrand factor can inhibit these ligand–integrin interactions, since the RGD-containing peptides bind directly to the gpIIb/IIIa integrin (Huang, Holt, Lukasiewics & Niewiarowski, 1987; D'Souza, Ginsberg, Lam & Plow, 1988).

Since platelet aggregation plays an important role in hemostasis and thrombosis, considerable interest has developed in the identification of natural and synthetic molecules that can mimic the RGD motif and bind to gpIIb/IIIa. Such molecules include synthetic peptides (Plow, Pierschbacher, Ruoslahti, Marguerie & Ginsberg, 1985; Pytela, Pierschbacher, Ginsberg, Plow & Ruoslahti, 1986) and monoclonal antibodies which are specific for the platelet integrin.

Three murine monoclonal antibodies have been described which bind specifically to gpIIb/IIIa. The IgM-κ antibody PAC-1 (Taub, Gould, Garsky, Ciccarone, Hoxie, Friedman & Shattil, 1989) binds to gpIIb/IIIa on activated platelets, while the OP-G2 (Tomiyama, Tsubakio, Piotrowicz, Kurata, Loftus & Kunicki, 1992) and LJ-CP3 (Niiya, Hodson, Bader, Byers-Ward, Koziol, Plow & Ruggeri, 1987) antibodies bind to nonactivated platelets. Each of these antibodies inhibits fibrinogen binding to gpIIb/IIIa. Moreover, the binding of these antibodies to the platelet integrin is inhibited by RGD-containing peptides. These monoclonal antibodies are specific for the fibrinogen binding site of gpIIb/IIIa and therefore could serve as 'molecular mimics' for fibrinogen. The specificity of PAC-1 for the IIb/IIIa integrin was found to reside largely in the heavy-chain third complementarity-determining region (CDR3). The CDR3 loop contains an Arg-Tyr-Asp (RYD) tripeptide within a sequence which is highly homologous to the segment encompassing the RGD recognition motif of fibrinogen. OP-G2 and LJ-CP3 antibodies have also incorporated the same germline D-gene which includes the RYD sequence (Tomiyama, Brojer, Ruggeri, Kieber-Emmons, Shattil, Smiltneck, Gorski, Kumar & Kunicki, 1992).

In order to examine the conformation of the RYD sequence within the CDR3 loop of the heavy chain of the OP-G2 antibody, we initiated a crystallographic analysis of the OP-G2 Fab fragment. OP-G2 Fab fragments retain binding specificity and inhibit fibrinogen-mediated platelet aggregation (Tomiyama, Tsubakio, Piotrowicz, Kurata, Loftus & Kunicki, 1992). Here we report the crystallization and preliminary diffraction analysis of the OP-G2 antigen-binding fragment.

Experimental

The murine OP-G2 IgG1-κ antibody was produced as described previously (Tomiyama, Honda, Furabayashi, Mizutani, Tsubakio, Kurata, Yonezawa & Tarui, 1988). Monoclonal IgG was purified from ascites by ammonium sulfate precipitation (50% saturated) followed by ion-exchange chromatography using DEAE–Sepharose equilibrated at pH 7.5 with 0.02 *M* Tris-HCl. The immunoglobulin fraction was eluted with a gradient to 0.3 *M* NaCl. Pooled fractions were dialyzed against 0.1 *M* Tris, pH 8.0, and applied to a Protein A–Sepharose column. Fractions containing IgG1 monoclonal antibody were eluted at pH 8.0 with 0.01 *M* Tris-HCl. For the preparation of Fab fragments, the IgG1 fraction was dialyzed at pH 7.4 against phosphate-buffered saline–0.002 *M* EDTA. The protein solution was adjusted to 4.5 mg ml^{-1} and β-mer-

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Table 1. *Data-collection statistics*

Resolution shell lower limit (Å)	Average reflection intensity, I	Average $I/\sigma(I)$	No. of reflections in $I/\sigma(I)$ ranges					No. of reflections	No. of observations	R_{sym}*
			< 2	< 5	< 10	< 20	> 20			
3.42	1453.7	46.7	102	261	299	651	4706	6021	31004	0.031
2.71	416.9	17.2	563	1000	1077	1367	1772	5786	22577	0.059
2.37	155.3	5.4	1490	1926	1410	727	108	5676	13267	0.117
2.15	106.6	3.5	2115	2273	994	207	9	5629	11695	0.162
2.00	77.6	2.4	2960	2112	448	29	1	5591	10668	0.209
Totals	455.7	15.5	7230	7572	4228	2981	6596	28703	89211	0.044

* $R_{sym} = \sum_h \sum_i |I_{hi} - \bar{I}_h| / \sum_h \bar{I}_h$, where I_{hi} is the scaled intensity of the ith measurement of reflection h or its equivalent and $\bar{I}_h$ is the average intensity of reflection h.

captoethanol was added to a final concentration of 0.01 *M*. The solution was warmed to 37 °C and papain (Sigma Chemical Company, St Louis, MO) was added at a ratio of 1:100 enzyme:antibody. The digestion was allowed to proceed for 4 h at 37 °C. The reaction was terminated by the addition of iodoacetate to a final concentration of 0.02 *M*.

OP-G2 Fab fragments were fractionated from Fc fragments by DEAE Sepharose chromatography. Finally, the Fab fraction was purified to homogeneity and a single isoelectric species by chromatofocusing. The Fab sample was applied to a PBE-94 column and eluted with Polybuffer 96 in a pH range of 8.3 to 5.9. Pooled fractions of the major Fab component were homogeneous as judged by SDS–PAGE and isoelectric focusing gels. Samples of OP-G2 Fab were dialyzed exhaustively at 4 °C against 0.1 *M* sodium acetate and 0.02% (*w*/*v*) sodium azide (pH 5.5) and concentrated to approximately 10 mg ml^{-1}.

Crystals suitable for X-ray diffraction analysis were grown at 22 °C by vapor diffusion in hanging drops (McPherson, 1982). Drops contained equal volumes (2 μl) of protein stock and reservoir solutions.

Results and discussion

In optimal crystallization conditions, the reservoir contained 24% (*w*/*v*) polyethylene glycol 1000, 0.02 *M* imidazole malate (pH 5.6) and 0.16 *M* NaCl. The crystals grew in the shape of trapezoidal prisms to a size of 0.3 × 0.3 × 0.5 mm in one week. Symmetry inspection using precession pictures taken with a Xuong–Hamlin multiwire area-detector system (Edwards, Nielsen & Xuong, 1988) indicated that the crystals belonged to the space group $P2_12_12$ with unit-cell dimensions a = 93.1, b = 83.8 and c = 53.7 Å. The space group was confirmed by inspection of three-dimensional data collected later. Assuming a molecular mass of 49000 daltons, the unit cell contained four molecules with one Fab molecule per asymmetric unit, and the volume per unit mass (V_m) was 2.16 Å^3 dalton^{-1} with 43% solvent content. These values correspond to those typical for other protein crystals (Matthews, 1968). The crystals diffract to 2 Å resolution and are stable in the X-ray beam for 3 days.

A complete intensity data set for the crystals, to a resolution of 2 Å, was collected with a Xuong–Hamlin multiwire area-detector system (Xuong, Nielsen, Hamlin & Anderson, 1985) using monochromatized Cu $K\alpha$ radiation from a Rigaku RU-200 rotating-anode X-ray generator (focal size = 0.5 × 1.0 mm), set at 50 kV and 100 mA. About 89000 intensity measurements of 28700 reflections out to 2.0 Å resolution were collected. Table 1 summarizes the data-collection statistics for the intensity data set.

Phase calculation and structure determination of the OP-G2 Fab molecule using molecular replacement methods are currently underway.

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