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Characterization of Complement C3 as a Glycyrrhizin (GL)-Binding Protein and the Phosphorylation of C3α by CK-2, Which Is Potently Inhibited by GL and Glycyrrhetinic Acid *In Vitro*

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The physiological interaction between glycyrrhizin (GL) and serum complement C3, and the inhibitory effects of GL, glycyrrhetinic acid (GA), and a GA derivative (oGA) on the phosphorylation of C3 by casein kinase 2 (CK-2), were investigated *in vitro*. C3 was found to be a GL-binding protein (gbP), because (i) of its high affinity for a GL-affinity HPLC column; and (ii) both GL and GA induce conformational changes in C3. At least four trypsin-resistant fragments (p30, p25, p18, and p15) were detected when the ^{32}P-labeled C3α was digested with trypsin in the presence of 100 μM GA. Two of these (p25 and p15) were immuno-precipitated with anti-C3a serum. Furthermore, it was found that C3a contains GL-binding domains, because (i) C3a (anaphylatoxin) could be selectively purified from the synovial fluids of patients with rheumatoid arthritis by GL-affinity column chromatography (HPLC); and (ii) purified human C3a has a high affinity for a GL-affinity column. In addition, C3α (p115) of C3 was effectively phosphorylated by CK-2 in the presence of poly-Arg (a CK-2 activator) *in vitro*. This phosphorylation was completely inhibited by 10 μM oGA, 30 μM GA, or 100 μM GL. Taken together, these results suggest that the GL-induced inhibition of the physiological activities of C3a and C3α may be involved in the anti-inflammatory effect of GL *in vivo*.

Key words: anaphylatoxin, anti-inflammatory effect, casein kinase 2, complement C3, GL-binding protein, glycyrrhizin.

Abbreviations: CK-2, casein kinase 2; A-kinase, cAMP-dependent protein kinase; C-kinase, Ca^{2+}- and phospholipid-dependent protein kinase; CD, circular dichroism; CBB R-250, Commassie Brilliant Blue R-250; C3α, α-polypeptide of C3; HPLC, high performance liquid chromatography; GA, glycyrrhetinic acid; GL, glycyrrhizin; oGA, GA derivative; gbP, glycyrrhizin-binding protein; PMSF, phenylmethyl sulfonyl fluoride; poly-Arg, poly L-arginine; SDS–PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; UV, ultraviolet.

Glycyrrhizin (GL) is present in large quantities in the roots and rhizomes of licorice, *Glycyrrhiza glabra* L., and is composed of a molecule of glycyrrhetinic acid (GA), which has a steroid-like structure, and two molecules of glucuronic acid. We have studied the physiological characteristics of the GL-binding proteins (gbPs) involved in inflammatory responses and the inhibitory effects of GL on the activities of gbPs *in vitro* (*1–7*). Previously, we reported that (i) mouse casein kinase 2 (CK-2) is selectively purified by GL-affinity column chromatography (HPLC) as a gbP (*1*); (ii) GL selectively inhibits the CK-2-mediated *in vitro* phosphorylation of functional gbPs, such as glucocorticoid receptor (*1*), soybean lipoxygenase 3 (*2*), *Habu* venom and human secretory type II-A phospholipases A2 (*3*, *4*), and HIV-1 enzymes [reverse transcriptase (*5*, *6*) and protease (*7*)]; and (iii) a GA derivative (oGA) is a potent inhibitor (at one-tenth the concentration of GL) of the CK-2-mediated phosphorylation of these gbPs *in vitro* (*1–7*).

Serum complement (C) plays an important role in many acute inflammatory processes. In particular, C3 is the most common in human serum (approx. 1.2 mg/ml) and is phosphorylated *in vitro* by various Ser/Thr-protein kinases, such as A-kinase (*8*), C-kinase (*8*, *9*) and casein kinases (CK-1 and CK-2) (*10*), although at different domains of the molecule with different biological effects. The C3α moiety of C3 is known to be phosphorylated by both A-kinase and C-kinase (*8*). This phosphorylation renders C3 more resistant to cleavage at the Arg^{77}-Ser^{78} bond by either trypsin or both the classical and alternative pathway convertases (*11*). In addition, casein kinase (with different sensitivities to specific inhibitors and specific antibodies of CK-1 and CK-2) released from activated human platelets phosphorylates both the α- and β-polypeptide chains of C3 and increases its susceptibility to cleavage by elastase (*12*). A subsequent effect of the phosphorylation of C3 by platelet casein kinase is to enhance the opsonization of immune complexes (*13*).

Although GA (*14*) is characterized as a potent inhibitor of the classical and alternative complement activation pathways, the physiological interactions of two anti-inflammatory compounds (GL and GA) with main target-

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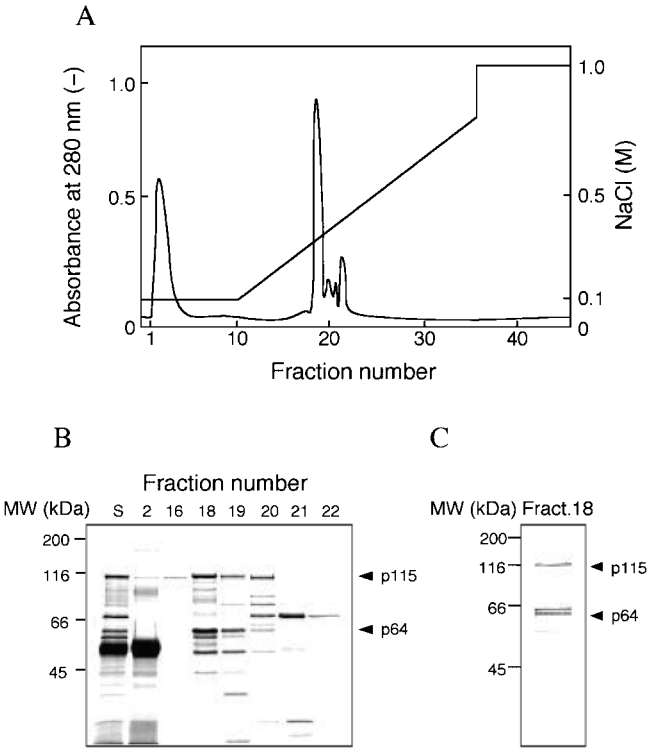

Fig. 1. **Purification of C3 by Mono Q column chromatography (HPLC).** A: The Superdex fraction prepared from guinea pig complement fraction (approx. 5 mg) was applied onto a Mono Q column, previously equilibrated with 20 mM Tris-HCl (pH 7.6) containing 0.1 M NaCl. Elution was carried out with a linear gradient of 0.1 to 0.8 M NaCl at a flow rate of 1.0 ml/min, and 1.0 ml fractions were collected. Absorbance at 280 nm (–). B: Polypeptides in the indicated fractions were analyzed by SDS–PAGE. The polypeptides on the gel were detected by staining with CBB R-250. S, Superdex fraction. C: Polypeptides in the Mono Q fraction (fraction 18) were detected mainly by Western blotting using anti-C3 serum.

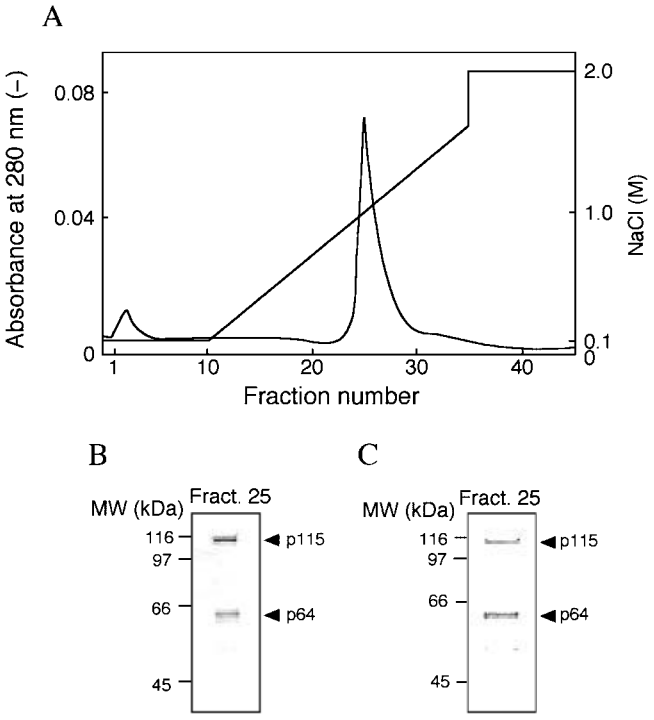

Fig. 2. **Characterization of C3 in the Mono Q fraction as a gbP by GL-affinity column chromatography (HPLC).** A: C3 in the Mono Q fraction was further purified on a GL-affinity column previously equilibrated with 20 mM MES-NaOH (pH 6.8) containing 0.1 M NaCl. Elution was carried out with a linear gradient of 0.1 to 1.5 M NaCl at a flow rate of 1.0 ml/min, and 1.0 ml fractions were collected. Absorbance at 280 nm (–). B: Polypeptides in the peak fraction (fraction 25) were analyzed by SDS–PAGE. The polypeptides on the gel were detected by CBB R-250. C: Polypeptides in fraction 25 detected by Western blotting using anti-C3 serum.

ing complements remain to be elucidated. The present study was carried out to determine the physiological interaction between C3 (from guinea pig and human sera) and GL, and the inhibitory effects of GL, GA, and oGA on the CK-2–mediated phosphorylation of C3 *in vitro*. Here, we describe (i) the further characterization of C3 as a gbP; (ii) the determination of the CD spectra of C3 incubated with either GA or GL; (iii) the inhibitory effects of GL, GA, and oGA on the CK-2-mediated phosphorylation of C3α (p115) *in vitro*; and (iv) biochemical experiments to determine the GL- and GA-binding domains of human C3a (anaphylatoxin).

MATERIALS AND METHODS

Chemicals—[γ-^{32}P]ATP (3,000 Ci/mmol) was obtained from Amersham Pharmacia Biotech (Arlington Heights, USA). Standard guinea pig complement (normal guinea pig serum) was obtained from Cedarlane (Ontario, Canada); human complement C3 (96% purity) from Biogenesis (Kingston, NH, USA); anti–human C3a rabbit serum (1:500–1:1,000 for Elisa) from Calbiochem (La Jolla, CA, USA); bovine pancreatic trypsin (approx. 3,000 NF units/mg) from Miles-Seravac (Maidenhead Berks, England); and phenylmethyl sulfonyl fluoride (PMSF) and a silver stain kit from Wako Pure Chemicals (Osaka). GL (20β-carboxyl-11-oxo-30-norolean-12-en-3β-yl-2-*O*-β-D-glucopyranuronosyl-β-D-glucopyranosiduronic acid, C42H62O16 = 822.92), GA (olean-12-en-3β-ol-11-one-30-oic acid) and oGA [olean-11,13(*18*)-diene-3β,30-diol-3β,30-di-*O*-hemiphthalate disodium salt] were kindly supplied by Minophagen Pharmaceutical (Tokyo).

Casein Kinase 2—Recombinant human CK-2 (rhCK-2) [a heterodimer of $\alpha_2\beta_2$ (α-subunit = 44 kDa and β-subunit = 26 kDa); specific activity: 400 units/μg] was obtained from Biomol Research Laboratories (Plymouth Meeting, PA, USA).

GL-Affinity Column—A GL-affinity column was prepared as originally described by Nakamura *et al.* (*15*) using Tresyl-5PW (packing gel for HPLC; Tosoh Mfg., Tokyo) and *N*-(glycyrrhizin)-30-α-lysine.

Partial Purification of C3 from Guinea Pig Complement Fraction—Complement C3 in the guinea pig complement fraction was partially purified by a combination of MonoQ column chromatography and gel filtration on a Superdex 200pg column, as reported previously (*16*).

Phosphorylation of Purified C3 by CK-2 In Vitro—The phosphorylation of C3 by CK-2 was assayed in reaction mixtures comprising 40 mM Tris-HCl (pH 7.6), CK-2 (approx. 10 ng), 20 μM [γ-^{32}P]ATP (500 cpm/pmol), 3 mM

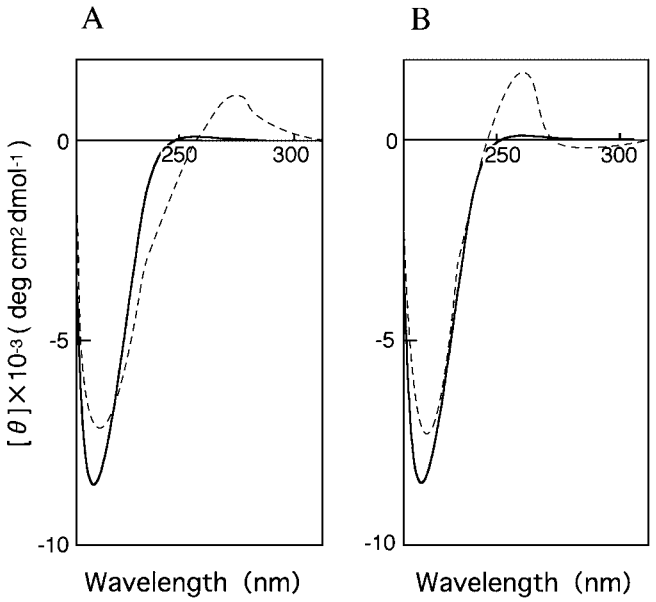

Fig. 3. **The conformational changes in C3 induced by GL and GA.** The far-UV CD spectra of purified C3 (approx. 4 μM) in the presence (.....) or absence (—) of either 100 μM GL (A) or 100 μM GA (B) in 20 mM MES-NaOH (pH 6.8) were measured at 25°C in the wavelength range 210–300 nm at a recording rate of 50 nm/min using a 10 mm cell in a J-720 CD spectropolarimeter (JASCO). Each spectral curve represents the mean of three scans.

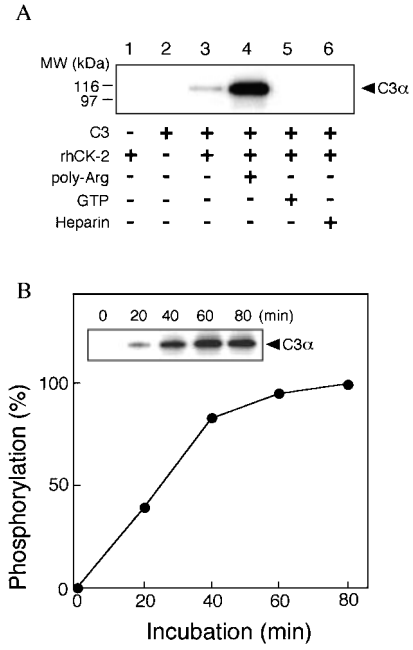

Fig. 4. **The CK-2-mediated phosphorylation of C3α (p115) and its kinetics.** A: Purified C3 (approx. 4 μg) was incubated for 30 min at 30°C with CK-2 (approx. 10 ng) and 20 μM [γ-^{32}P]ATP (500 cpm/pmol) and poly-Arg (0.5 μg). Phosphorylated C3α (p115) in the reaction mixtures was detected by SDS–PAGE followed by autoradiography. CK-2 alone (lane 1); lane 2, purified C3 fraction alone; lane 3, lane 2 + CK-2; lane 4, lane 3 + poly-Arg; lane 5, lane 4 + 50 μM GTP; and lane 6, lane 4 + heparin (0.2 μg). B: Purified C3 was incubated for the indicated periods at 30°C with CK-2 (approx. 10 ng) and 20 μM [γ-^{32}P]ATP (500 cpm/pmol) in the presence of poly-Arg (0.5 μg). The degree of phosphorylation of C3α (p115) on the X-ray film was measured with a spectrophotometer. 100% represents maximum phosphorylation of C3α by CK-2 after incubation for 80 min at 30°C.

Mn^{2+}, 2 mM DTT, 0.5 μg of poly-Arg (a CK-2 activator), and 4 μg of purified guinea pig or human C3 (a protein substrate). After incubation for 30 min at 30°C, ^{32}P-labeled C3α (p115) of C3 in the reaction mixtures was detected directly by autoradiography after SDS–PAGE, as reported previously (*1–7*).

RESULTS

Further Purification of Guinea Pig C3 as a gbP—To purify C3 in the Superdex fraction prepared from guinea pig complement fraction, the Superdex fraction (approx. 5 mg protein) was applied onto a Mono Q column and eluted with a linear gradient of 0.1 to 0.8 M NaCl (Fig. 1A). SDS–PAGE revealed two polypeptides (115 and 64 kDa) that crossreacted with anti-C3 serum in the indicated fractions (Fig. 1, B and C). The molecular sizes of these two polypeptides (115 and 64 kDa) in the Mono Q fraction correspond to those reported for C3α (115 kDa) and C3β (65 kDa) purified from guinea pig serum (*17*).

To purify C3 further from the Mono Q fraction, fraction 18 (Fig. 1A) was applied to a GL-affinity column. Elution was carried out with a linear gradient of 0.1 to 1.5 M NaCl. C3 eluted from the GL-affinity column as a single polypeptide between 0.9 and 1.2 M NaCl (Fig. 2A). SDS–PAGE detected two main polypeptides (115 and 64 kDa) that crossreacted with anti-C3 serum (Fig. 2, B and C). These results show that guinea pig C3 [α (p115) and β (p64)] has a high binding affinity for a GL-affinity column.

Conformational Changes in C3 Induced by GL and GA—To obtain further confirmation of the direct binding of GL or GA to C3, the CD spectra of C3 were determined after incubation for 10 min at room temperature with or without either GL or GA. The negative peak at 215nm shifted to 223 nm and a positive peak at 265 nm appeared after incubation of C3 with 100 μM GL (Fig. 3A). With 100 μM GA, the negative peak shifted to 217nm and a positive peak appeared at 257 nm (Fig. 3B). These results suggest that both GL and GA bind directly to C3, and this binding induces conformational changes in C3 *in vitro*.

Effects of GL, GA and oGA on the CK-2-Mediated Phosphorylation of C3α in Vitro—Since it has been reported that the α-polypeptide (C3α) of C3 functions as an effective phosphate acceptor for CK-2 *in vitro* (*11–13*), similar experiments were performed using purified guniea pig C3 as a phosphate acceptor of CK-2 *in vitro*. It was confirmed that (i) C3α (p115) of purified guniea pig C3 was phosphorylated by CK-2 (lane 3, Fig. 4A); and (ii) this phosphorylation was greatly stimulated by poly-Arg (lane 4), but inhibited by 50 μM GTP (lane 5) or heparin (lane 6) *in vitro*. In the presence of poly-Arg (a CK-2 activator), the phosphorylation of C3α by CK-2 increased in a time-dependent manner until 40 min and reached a plateau within 80 min (Fig. 4B).

The effects of GL, GA and oGA on the CK-2–mediated phosphorylation of C3α were examined *in vitro*. The phosphorylation of C3α (p115) by CK-2 was completely

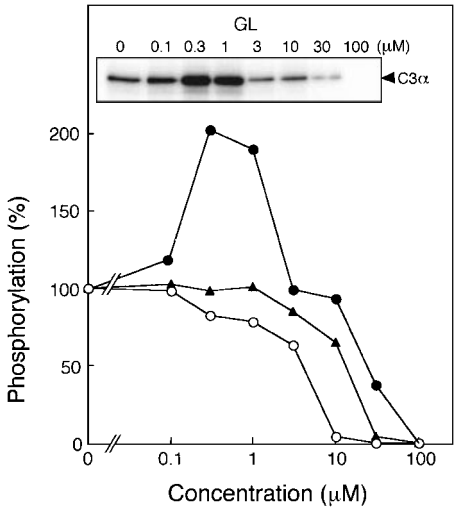

Fig. 5. **The effects of GL, GA, and oGA on the CK-2-mediated phosphorylation of C3α *in vitro*.** Purified C3 (approx. 4 μg) was incubated for 30 min at 30°C with CK-2 (approx. 10 ng) and 20 μM [γ-^{32}P]ATP (500 cpm/pmol) in the presence of the indicated amounts of either GL, GA or oGA. The ^{32}P-labeled C3α (p115) in the reaction mixtures was detected by autoradiography after SDS–PAGE. The autoradiogram (inset) was scanned with a spectrophotometer. Solid circle, GL; solid triangle, GA; and open circle, oGA. 100% represents the CK-2–mediated phosphorylation of C3α determined in the absence of these three compounds.

inhibited by 10 μM oGA, 30 μM GA, or 100 μM GL (Fig. 5). Interestingly, GL between 0.3 and 1.0 μM stimulated the CK-2–mediated phosphorylation of C3α by about 2-fold (Fig. 5). These results show that (i) CK-2 effectively phosphorylates C3α (p115) in the presence of a CK-2 activator, such as poly-Arg, *in vitro*; and (ii) this phosphorylation is completely inhibited by GL at approx. 100 μM, but stimulated significantly at lower concentrations (0.3–1 μM).

Detection of Trypsin-Resistant Fragments Containing the GL- and GA-Binding Domains of C3α—To detect the GA- and GL-binding domains in the C3α molecule, purified C3 was preincubated for 60 min at 30°C with CK-2 and 20 μM [γ-^{32}P]ATP (500 cpm/pmol) in the presence of 0.5 μM GL and poly-Arg (0.5 μg/ml). The ^{32}P-labeled C3α (p115) of C3 was digested with trypsin in the presence or absence of either 1 μM GL, 100 μM GA, or 100 μM GL. In the absence of these two compounds, ^{32}P-labeled C3α was digested by trypsin into small fragments in a time-dependent manner (Fig. 6A, left side). However, there was no obvious difference in the pattern of fragments generated from ^{32}P-labeled C3α by limited digestion with trypsin in the presence or absence of 1 μM GL (Fig. 6A). In contrast, there were several distinct trypsin-resistant fragments with relatively high molecular sizes (p80, p42, p40, and p30) in the presence of 100 μM GL (Fig. 6B). In the presence of 100 μM GA, at least four relatively low molecular size trypsin-resistant fragments (p30, 5, p18, and p15) were mainly detected (Fig. 6C). No effects of GL or GA at 300 μM on the activity of trypsin were detected (results not shown). Interestingly, two fragments (p25 and p15) generated from ^{32}P-labeled C3α digested with trypsin in the presence of 100 μM GA were immunoprecipitated with anti-C3a serum (Fig. 6C). These results suggest that two fragments (p25 and p15) generated from C3α (p115) by limited digestion with trypsin may include C3a (anaphylatoxin), containing the GA-binding domains.

Characterization of Human C3a as a gbP—To confirm that C3a (human anaphylatoxin: 77 amino acid residues) is a gbP, attempts were made to purify C3a selectively from the heparin-binding fraction prepared from the synovial fluids of patients with rheumatoid arthritis using a GL-affinity HPLC column. Several proteins, including p33, p28, and p9, eluted between 0.8 and 1.3 M NaCl from the GL-affinity column (Fig. 7). The sequence of the 20 N′-terminal amino acids (SVQLTEKRMDKVGKYPELR) of p9 is identical to the corresponding sequence of human C3a (anaphylatoxin) (*18*). The sequence of the 12 N′-terminal amino acids (EDLCRAPDGKKG) of p33 and the 20 N′-terminal amino acids (NTGCYGIPGMPGLPGAPGKD) of p28 are identical to the corresponding sequences of human C1qA and C1qC (*19*), respectively. Under the same experimental conditions, purified human C3a (approx. 9 kDa) eluted between 0.8 and 1.2 M NaCl from a GL-affinity column as a single peak (Fig. 8A). SDS–PAGE detected a single protein with a molecular size of approx 9 kDa in the peak fractions (Fig. 8B). These results show that (i) human C3a is a gbP; and (ii) two fragments (C1qA and C1qC) generated from C1 have binding affinity for a GL-affinity column.

DISCUSSION

In the present study, C3 highly purified from guinea pig serum was found to be a GL-binding protein (gbP), since (i) it had a high binding affinity for a GL-affinity column (Fig. 2A); and (ii) both GL and GA induced significant conformational changes in C3 (Fig. 3). Similar results were obtained with human C3 (1,663 amino acid residues), which has about 76.8% homology with guinea pig C3 (1,666 amino acid residues). In addition, at least four trypsin-resistant fragments (p30, p25, p18, and p15) were detected when ^{32}P-labeled C3α (p115) of C3 was digested with trypsin in the presence of 100 μM GA (Fig. 6C). Two of these (p25 and p15) were immuno-precipitated with anti-human C3a serum (Fig. 6D). These results suggest that the GA moiety of the GL molecule may be responsible for its binding to C3α, which contains multiple basic amino acid-rich domains. Furthermore, human C3a (anaphylatoxin) in the heparin-affinity fraction prepared from the synovial fluids of patients with rheumatoid arthritis was selectively purified by GL-affinity column chromatography (Fig. 7). The high binding affinity of purified human C3a for a GL-affinity column was clearly confirmed (Fig. 8). Taken together, these results suggest that C3a (from guinea pig and human sera) includes the GL- and GA-binding domains of C3.

C3α (p115) of guinea pig C3 was found to be phosphorylated by CK-2 (Fig. 4), as has been reported by Nilsson *et al.* (*11*, *12*). Under the same experimental conditions, a similar phosphorylation by CK-2 was observed with human C3 (data not shown). This phosphorylation was inhibited by GL (ID_{50} = approx. 30 μM) as well as GA (ID_{50} = approx. 20 μM) in a dose dependent manner (Fig.

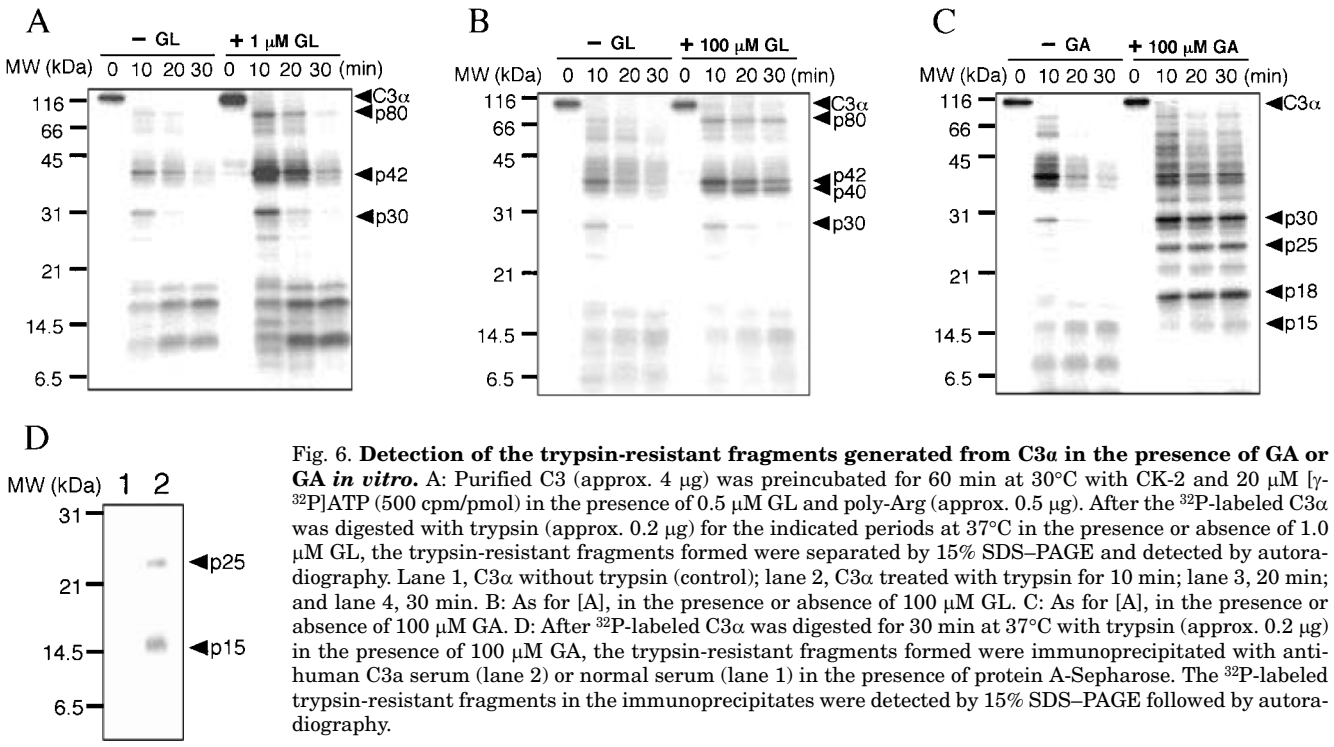

Fig. 6. **Detection of the trypsin-resistant fragments generated from C3α in the presence of GA or GA *in vitro*.** A: Purified C3 (approx. 4 μg) was preincubated for 60 min at 30°C with CK-2 and 20 μM [γ-^{32}P]ATP (500 cpm/pmol) in the presence of 0.5 μM GL and poly-Arg (approx. 0.5 μg). After the ^{32}P-labeled C3α was digested with trypsin (approx. 0.2 μg) for the indicated periods at 37°C in the presence or absence of 1.0 μM GL, the trypsin-resistant fragments formed were separated by 15% SDS–PAGE and detected by autoradiography. Lane 1, C3α without trypsin (control); lane 2, C3α treated with trypsin for 10 min; lane 3, 20 min; and lane 4, 30 min. B: As for [A], in the presence or absence of 100 μM GL. C: As for [A], in the presence or absence of 100 μM GA. D: After ^{32}P-labeled C3α was digested for 30 min at 37°C with trypsin (approx. 0.2 μg) in the presence of 100 μM GA, the trypsin-resistant fragments formed were immunoprecipitated with anti-human C3a serum (lane 2) or normal serum (lane 1) in the presence of protein A-Sepharose. The ^{32}P-labeled trypsin-resistant fragments in the immunoprecipitates were detected by 15% SDS–PAGE followed by autoradiography.

5). Interestingly, The CK-2–mediated phosphorylation of C3α was significantly stimulated by GL between 0.3 and 1.0 μM (Fig. 5). This stimulation may be due to the binding of GL to CK-2, which has been reported in the CK-2–mediated *in vitro* phosphorylation of other gbPs, such as glucocorticoid receptor (*1*), soybean lipoxygenase 3 (*2*), secretory type II-A phospholipase A_2 (*3*) and HIV-1 enzymes [reverse transcriptase (*5*, *6*) and protease (*7*)].

Nilsson *et al.* reported that a casein kinase released from activated platelets phosphorylates both the α- and β-polypeptide chains of C3, and that there is an increase in its susceptibility to cleavage by elastase (*12*). The phosphorylation of C3α by platelet casein kinase enhances the opsonization of platelet immune complexes (*13*). Therefore, it seems that the GL-induced stimulation of the CK-2–mediated phosphorylation of C3α at lower concentrations (0.3–1 μM) may be involved in an enhancement of the opsonization of platelet immune complexes. Since GL at low levels induces interferon-γ production (*22*) as well as extra T cell differentiation (*23*), the GL-induced stimulation of the CK-2–mediated phosphorylation of cellular functional proteins, including protein kinases and transcriptional factors (*1*, *2*, *24*, *25*), may be a common event involved in these GL-induced biological effects *in vivo*.

In the complement-mediated haemolysis assay, the inhibitory effect of GA, but not GL, appears most profound on C2 (*14*). Our observations presented here suggest that C3 is one of the main targets of GA: (i) GA induces significant conformational changes in C3 purified from the complement fraction of guinea pig serum (Fig. 3B); (ii) there are at least four trypsin-resistant fragments (p30, p25, p18, and p15) generated from C3α of C3 by limited digestion with trypsin in the presence of 100 μM GA (Fig. 6C); and (iii) GA at concentrations above 20 μM completely inhibits the CK-2–mediated phosphorylation of C3α *in vitro* (Fig. 5). Also, it was found that C3a contains the GL-binding domains, because purified human C3a had a high binding affinity for a GL-affinity column (Fig. 8). Indeed, C3a in the heparin-binding fraction prepared from the synovial fluids of patients with rheumatoid arthritis was selectively purified by GL-affinity column chromatography (Fig. 7). Therefore, it is concluded that C3a has a high affinity for these two anti-inflammatory compounds (GL and GA), as has been demonstrated for other basic gbPs, such as high mobility group protein 1 (*20*) and bovine lactoferrin (*21*). The high binding affinities of GL and GA for C3α and its proteolytically degraded fragments (including C3a) may be involved in their inhibitory effects on their C3-mediated inflammatory responses *in vivo*. Since both GL and GA at 100 μM completely inhibit the CK-2-mediated phosphorylation of C3α by CK-2 (Fig. 5) as well as other two protein kinases (A-kinase and C-kinase; data not shown), the GL- and GA-induced inhibitions of the physiological activities of C3 and C3a may partly explain their anti-inflammatory effects *in vivo*.

To understand clearly the anti-inflammatory effects of GL and GA in complement-mediated inflammatory processes, further analytical studies are required (i) to determine their specific binding domains on both C3α and C3a; and (ii) to characterize these two compounds (GL and GA) as potent inhibitors of the biological activities of the GL- and GA-binding fragments (C1qA, C1qC, C3a, C4a, and C5a generated proteolytically from their precursor complements) during immunological responses, including inflammation.

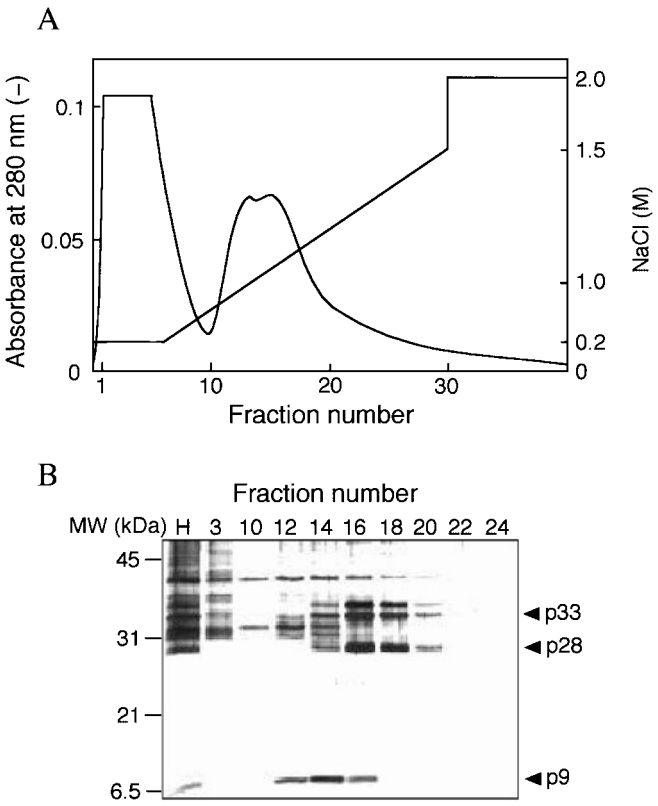

Fig. 7. Selective purification of human C3a as a gbP from the heparin fraction prepared from the synovial fluids of patients with rheumatoid arthritis by GL-affinity column chromatography. A: The heparin-affinity fraction (approx. 2.0 mg protein) prepared from the synovial fluids of patients with rheumatoid arthritis was applied onto a GL-affinity HPLC column, equilibrated previously with 20 mM HEPES buffer (pH 7.6) containing 5 mM EDTA and protease inhibitors (0.1 mM PMSF, 0.5 mM amino-benzamidine, and 1 μg/ml leupeptin). Elution was carried out with a linear gradient of 0.2 to 1.5 M NaCl at a flow rate of 1.0 ml/min at 4°C; 1.0 ml fractions were collected. Absorbance at 280 nm (–). Polypeptides in the indicated fractions analyzed by 15% SDS–PAGE with silver staining. H, heparin-affinity fraction.

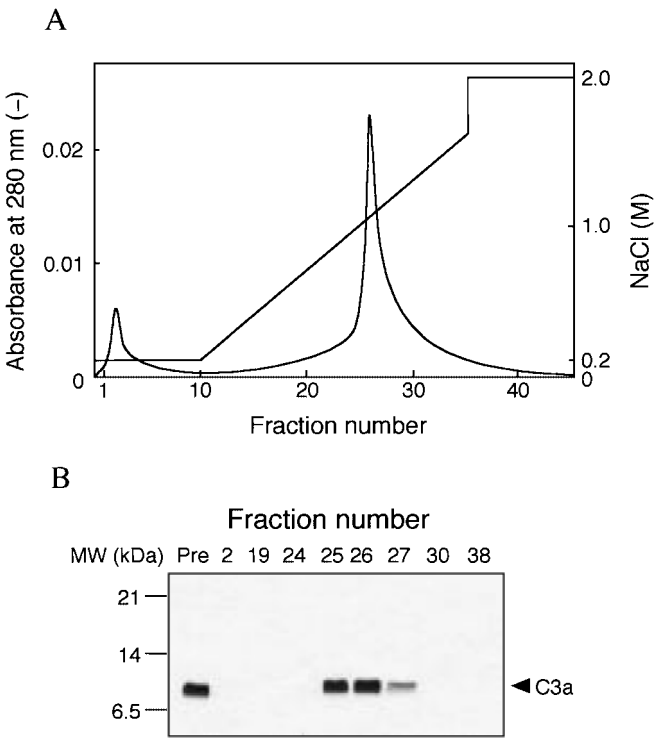

Fig. 8. **Characterization of human C3a as a gbP.** A: Purified human C3a (approx. 50 μg protein) was applied onto a GL-affinity HPLC column, equilibrated previously with 20 mM MES buffer (pH 6.8) containing 5 mM EDTA and 0.2 M NaCl. Elution was carried out with a linear gradient of 0.2 to 1.5 M NaCl at a flow rate of 1 ml/min at room temperature and 1.0 ml fractions were collected. Absorbance at 280 nm (–). B: Polypeptides in the indicated fractions detected by silver staining after 17.5% SDS–PAGE.

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