Biochemical Characterization of the Penta-EF-hand Protein
Grancalcin and Identification of L-plastin as a Binding Partner*

Received for publication, February 1, 2001
Published, JBC Papers in Press, February 13, 2001, DOI 10.1074/jbc.M100965200

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Grancalcin is a recently described Ca\(^{2+}\)-binding protein especially abundant in human neutrophils. Grancalcin belongs to the penta-EF-hand subfamily of EF-hand proteins, which also comprises calpain, sorcin, peflin, and ALG-2. Penta-EF-hand members are typified by two novel types of EF-hands: one that binds Ca\(^{2+}\) although it has an unusual Ca\(^{2+}\) coordination loop and one that does not bind Ca\(^{2+}\) but is directly involved in homodimerization. We have developed a novel method for purification of native grancalcin and found that the N terminus of wild-type grancalcin is acetylated. This posttranslational modification does not affect the secondary structure or conformation of the protein. We found that both native and recombinant grancalcin always exists as a homodimer, regardless of the Ca\(^{2+}\) load. Flow dialysis showed that recombinant grancalcin binds two Ca\(^{2+}\) per subunit with positive cooperativity and moderate affinity (IC\(_{50}\) of 25 and 85 \(\mu\)M in the presence and absence of octyl glycoside, respectively) and that the sites are of the Ca\(^{2+}\)-specific type. Furthermore, we showed, by several independent methods, that grancalcin undergoes important conformational changes upon binding of Ca\(^{2+}\) and subsequently exposes hydrophobic amino acid residues, which direct the protein to hydrophobic surfaces. By affinity chromatography of solubilized human neutrophils on immobilized grancalcin, L-plastin, a leukocyte-specific actin-binding protein, was found to interact with grancalcin in a negative Ca\(^{2+}\)-dependent manner. This was substantiated by co-immunoprecipitation of grancalcin by anti-L-plastin antibodies and vice versa.

Grancalcin is a recently described protein present in some cells of hematopoietic origin and especially abundant in human neutrophils (1–3). Grancalcin belongs to the calpain subfamily of EF-hand Ca\(^{2+}\)-binding proteins, comprising calpain light and heavy chain, sorcin, grancalcin, ALG-2, peflin, and YG25-yeast (4, 5). So far, the members of this subfamily seem to have diverse functions: calpain functions as a protease and can regulate adhesion, sorcin binds to and regulates the cardiac ryanodine calcium channel, ALG-2 plays a role in apoptosis, and the function of grancalcin is unknown. From amino acid sequence comparison, it was initially deduced that these proteins possess four EF-hand motifs (6). However, recent studies on the three-dimensional structure of dVI, the Ca\(^{2+}\)-binding domain of calpain light chain (7, 8), surprisingly revealed the presence of a fifth EF-hand N-terminal to the previously identified EF-hands. This EF-hand is of a novel type and has therefore escaped detection by sequence similarity analysis. This group of proteins is thus characterized by five EF-hands and has accordingly been named the penta-EF-hand (PEF)\(^{1}\) subfamily (4). In addition, the members of the PEF subfamily contain N termini of varying length and sequence but all rich in glycine and hydrophobic residues. In calpain, EF1 (although of an unusual sequence composition) and EF2 are paired and bind two Ca\(^{2+}\) with high affinity. EF3 and EF4 are also paired and possess one site of high affinity and one of low affinity for Ca\(^{2+}\). Finally, EF5, which does not bind Ca\(^{2+}\), is paired with a similar EF5 of another monomer and is thus a dimerization module. Native calpain is a heterodimer of the calpain light and heavy chain (9). The recent crystalization of apograncalcin (10, 11) has shown great overall similarity to the crystal structure of calpain. Grancalcin was also found to form dimers by paring of cognate EF\(^{5}\)s. Unfortunately, grancalcin precipitated in the presence of Ca\(^{2+}\) and the topology of active Ca\(^{2+}\)-binding sites could not be determined, except for binding of Ca\(^{2+}\) to EF3 from one monomer. Moreover, the N terminus of grancalcin is disordered in the crystals, and the first well defined residue is Ser\(^{16}\). In molecular sieve chromatography, purified grancalcin migrates as a homodimer (1) and ALG-2 as a monomer (12, 13). However, when using a chemical cross-linker, ALG-2 could be shown to exist also as a dimer (13).

The cDNA for grancalcin is 1.65 kilobase pairs long and contains an open reading frame for 217 amino acids; the first 14 amino acids have been reported to be removed posttranslationally to give rise to a 203-residue-long functional protein (2). This presumably functional grancalcin has a calculated molecular mass of 22.4 kDa, while grancalcin migrates as a 28-kDa protein in SDS-PAGE (1, 2). The reason for this discrepancy is unknown, but it is not due to glycosylation (1). Grancalcin translocates to membranes upon binding of Ca\(^{2+}\) (2, 3, 14). This is a common feature of several EF-hand proteins. Translocation is often mediated by exposure of hydrophobic patches of amino acids subsequent to Ca\(^{2+}\) binding, as is the case for calpain (15) and calretinin (16). In other instances, such as in the recoverin family, a covalently bound fatty acid becomes exposed after

\(^{1}\) The abbreviations used are: PEF, penta-EF-hand; OG, octyl-\(\beta\)-glucoside; PAGE, polyacrylamide gel electrophoresis; PIPES, 1,4-piperazinediethanesulfonic acid; FPLC, fast protein liquid chromatography; HPLC, high pressure liquid chromatography; DSG, disuccinimidyl glutarate.
Ca\textsuperscript{2+}-induced conformational changes, and this leads to translocation of the protein to membranes, a mechanism also called the myristoyl switch (17). Which of these molecular schemes prevails for grancalcin is at present unknown.

EF-hand proteins have affinities for Ca\textsuperscript{2+} varying from 10\textsuperscript{8} to 10\textsuperscript{9} M\textsuperscript{-1}. Moreover, some show great selectivity toward Ca\textsuperscript{2+}, such as calmodulin, S100 proteins, and calretinin, whereas others also bind Mg\textsuperscript{2+}, such as parvalbumin and recoverin (for a review, see Ref. 18). Neither the affinity nor the selectivity can be deduced from the sequence of the EF-hands. Except for a positive \(40\) Ca\textsuperscript{2+} overlay, no information on the ion-binding and conformational changes of grancalcin is available.

Because the biochemical characterization of grancalcin has been very sparse and nothing is known about the function of grancalcin, we decided to address these issues. In this report, we have identified a N-terminal posttranslational modification of wild-type grancalcin by mass spectroscopy and sequencing. Because grancalcin precipitates in the presence of Ca\textsuperscript{2+} in most experiments, we have generated three mutants of grancalcin with varying N-terminal deletions. We show that these mutants do not precipitate to the same degree as recombinant grancalcin. We have also compared critical properties of wild-type and recombinant grancalcin, determined the binding characteristics of grancalcin for Ca\textsuperscript{2+} by flow dialysis, and probed cation induced conformational changes by fluorometry of the intrinsic Trp residues. Finally, using affinity chromatography, we identified the actin-bundling protein L-plastin as binding partner of grancalcin and document the Ca\textsuperscript{2+} dependence of this interaction.

**EXPERIMENTAL PROCEDURES**

**Isolation of Human Neutrophils—**Human neutrophils were isolated from volunteer donors as described (19). In short, erythrocytes were sedimented by 2% dextran (Amersham Pharmacia Biotech) in saline, and the leukocyte-rich supernatant was submitted to 400 × g density centrifugation for 30 min on Lymphoprep (Nycomed, Oslo, Norway).

The pellet was submitted to hypotonic lysis of contaminating erythrocytes for 30 s in pure water, after which toxicity was restored by the addition of NaCl. The neutrophils were washed once, counted, and resuspended in saline.

**SDS-PAGE and Immunoblotting—**SDS-PAGE (20) and immunoblotting (21) were performed on Bio-Rad systems according to the instructions given by the manufacturer (Bio-Rad) and as described (3), except for visualization of immunoblots, which was by metal-enhanced diaminobenzidine tetrahydrochloride (Pierce).

**Protein Measurement—**Crude protein concentration was determined by the Lowry method (22) according to the instructions given by the manufacturer (Bio-Rad), and catalase ranging from 0.05 to 0.5 mg/ml was used as a standard. Grancalcin was quantified by enzyme-linked immunosorbent assay as previously described (3). Purified grancalcin was quantified by the ultraviolet absorption spectrum using a molar extinction coefficient ε\textsubscript{280} of 28,000 M\textsuperscript{-1} cm\textsuperscript{-1}.

**Subcellular Fractionation of Human Neutrophils—**Isolated neutrophils at 3 × 10\textsuperscript{6} cells/ml were incubated in saline with 5 mM diisopropyl fluorophosphate (Sigma) for 5 min on ice and centrifuged at 200 × g for 6 min. Cell pellets were resuspended at 2 × 10\textsuperscript{6} cells/ml in binding buffer (100 mM KCl, 3 mM NaCl, 10 mM PIPES (pH 7.2)) containing 0.5 mM phenylmethylsulfonyl fluoride, 200 units/ml aprotinin, and 100 μl of matrix solution (n-cyano-4-hydroxycinnamic acid in acetonitrile/methanol; Hewlett Packard), and 0.5 μl of the mixture was applied to the probe and allowed to dry. The purified proteins (wild-type grancalcin and recombinant grancalcin) were diluted 4–10 times with a 20 mM octyl-β-d-glucopyranoside (OG) (Roche Molecular Biochemicals) solution in Tris-Cl (pH 7.8) including 10% acetonitrile for 3 h at 37 °C. The reaction was stopped by the addition of 400 μl of 1% trifluoroacetic acid. The resulting fragments were purified by HPLC on a 21.1 × 150-mm C-8 column (Vydac, Hespera, CA) as described above.

**Mass Spectrometry and Sequence Analysis—**The purified peptides were analyzed in a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (Biflex, Bruker-Franzen, Bremen, Germany). For analysis, 0.5 μl of the sample was mixed with 0.5 μl of matrix solution (n-cyano-4-hydroxycinnamic acid in acetonitrile/methanol; Hewlett Packard), and 0.5 μl of the mixture was applied to the probe and allowed to dry. The purified proteins (wild-type grancalcin and recombinant grancalcin) were diluted 4–10 times with a 20 mM octyl-β-d-glucopyranoside (OG) (Roche Molecular Biochemicals) solution in Tris-Cl (pH 7.8) including 10% acetonitrile for 3 h at 37 °C. The reaction was stopped by the addition of 400 μl of 1% trifluoroacetic acid. The resulting fragments were purified by HPLC on a 21.1 × 150-mm C-8 column (Vydac, Hespera, CA) as described above.

**Proteolytic Cleavages—**For the identification of the N-terminal modification in wild type grancalcin, 20 μg of both wild type and recombinant grancalcin was incubated with 0.5 μg of endoproteinase Glu-C (Roche Molecular Biochemicals) in 500 μl 50 mM NH\textsubscript{4}HCO\textsubscript{3}, (pH 7.8) including 10% acetonitrile for 20 h at room temperature. The reaction was stopped by the addition of 1 ml 1% trifluoroacetic acid. The resulting fragments were purified by HPLC on a 21.1 × 150-mm C-8 column (Vydac, Hespera, CA) as described above.

**Chemical Cross-linking—**Proteins were cross-linked with the chemical cross-linking agent DSG (Pierce) essentially as described (13). In short, proteins were incubated for 30 min at room temperature in 100 μl of cross-linking buffer (20 mM HEPS (pH 8.0), 50 mM NaCl, and 1 ml of dithiothreitol) to which 5 μl of DSG was added (from 10 mM stock in N,N-Dimethylformamide). The reaction was quenched by the addition of 125 μl of 1 M Tris-HCl, pH 7.5, and cross-linking was evaluated by SDS-PAGE.
plemented with 1 mM EGTA, dialyzed overnight against buffer A containing 0.1 mM EGTA, and passed through a Sephadex G25 column (0.8 × 40 cm) equilibrated in buffer A (50 mM Tris-HCl buffer (pH 7.5), 150 mM KCl). The protein contained less than 0.1 mol of Ca$^{2+}$/mol. Ca$^{2+}$-binding was measured on the recombinant protein at 25 °C by the flow dialysis method (23). Protein concentrations were measured by the method of Bradford. Processing of data and evaluation of the binding constants were as described (18).

Optical Methods to Probe the Tryp Environment—Emission fluorescence spectra were obtained in a PerkinElmer Life Sciences LS-5B spectrophotometer. The measurements were carried out at 25 °C on 4 μM protein in 50% acetonitrile in 100 mM AB followed by shrinkage in acetonitrile at 0 °C. Excess liquid was removed, and the gel pieces were washed for 1 h in an ice bath and allowed to swell in 25 mM AB including 2 mM phenethylsulfonyl fluoride, 1 mM phenylmethylsulfonyl fluoride, 200 units/ml aprotinin, 100 μg/ml leupeptin, and 0.1 mM EGTA and incubated overnight at 4 °C. Unsolubilized material were spun down (5000 × g for 10 min), and 10 μl of supernatant, supplemented with 0.5 mM EGTA, were applied to each to each of the three columns. The columns were washed extensively in binding buffer plus 0.5 mM EGTA, and bound proteins were eluted with binding buffer plus 5 mM CaCl$_2$. Fractions of 1 ml were collected and evaluated by SDS-PAGE. Fractions with a visible protein band were pooled and concentrated by centrifugation and subjected to SDS-PAGE. The identified band was cut out of the SDS gel and digested with trypsin essentially as described by Wilm et al. (24). In short, the gel piece was cut into small cubes and washed for 1 h in 100 mM NH$_4$HCO$_3$ (AB), and excess liquid was removed. Then the proteins were reduced for 30 min at 60 °C in 100 μl of 4 mM dithiothreitol in 100 mM AB, cooled, and alkylated by the addition of 10 μl of 100 mM iodoacetamide followed by a 30-min incubation at room temperature in the dark. Excess liquid was removed, and the gel pieces were washed for 1 h in 50% acetonitrile in 100 mM AB followed by shrinkage in acetonitrile and vacuum centrifugation. The tube with the dry gel pieces was placed in an ice bath and allowed to swell in 25 mM AB. After 45 min, the excess liquid was removed, and buffer was added to cover the gel pieces during overnight incubation at 37 °C. The next day, the liquid was removed and combined with two consecutive extractions with 50 μl of 0.1% trifluoroacetic acid in 60% acetonitrile. The extract was dried in a vacuum centrifuge to near dryness followed by the addition of 5 μl of 0.1% trifluoroacetic acid in 30% acetonitrile. For analysis, 0.4 μl of the sample was mixed with 5 μl of the standard mixture (angiotensin II and cytochrome c). After 0.06 and 1 pmol/μl, respectively) and 0.5 μl matrix solution (a-cyano-4-hydroxy-cinnamic acid) and measured by mass spectrometry as described above, except the instrument was upgraded with the time lag focusing option (delayed extraction). The peptide molecular mass fingerprint was used for a search of the SWISS-PROT and TrEMBL data base, using the "PeptIdent" tool on the ExPaSy Molecular Biology Server of the Swiss Institute of Bioinformatics (25).

Immunoprecipitation—Cytosol from human neutrophils (prepared as described above) was diluted with binding buffer to a final concentration of 1 mg/ml (as determined by Bio-Rad). 5 μl of anti-grancalcin or anti-L-plastin antibodies (two different antibodies; LPLA1A1, which binds to L-plastin irrespective of Ca$^{2+}$ load, and LPL7.2, which only binds to the Ca$^{2+}$-loaded form of L-plastin (described in Ref. 26), a kind gift from Dr. Eric J. Brown (Division of Infectious Disease, Howard Hughes Medical Institute, St. Louis, MO)) were added to 1 ml of the diluted cytosol in the presence of 0.5 mM CaCl$_2$ or 5 mM EGTA, respectively. The solution were rotated end over end overnight at 4 °C. Next, 100 μl of protein A-Sepharose beads (Amersham Pharmacia Biotech), prepared in binding buffer as described by the manufacturer, were added and rotated end over end for 2 h at 4 °C. The tubes were centrifuged to pellet beads, and the supernatant was gently removed. The pellets were washed three times in binding buffer with either 0.5 mM CaCl$_2$ or 5 mM EGTA present before reconstitution into the initial volume. Samples were run on SDS-PAGE and immunoblotted as described above. For immunoblotting with L-plastin and MRP14 antibodies, the following dilutions were used: LPLA1A1 primary antibody, 1:1000; secondary antibody (goat anti-mouse (Dako A/S, Glostrup, Denmark), 1:1000; LPL7.2 primary antibody, 1:1000; secondary antibody (rabbit anti-rat (Dako A/S), 1:1000; MRP14 primary antibody (Bachem, Bubendorf, Switzerland), 1:2000 (biotinylated); secondary layer (avidine horseradish peroxidase (P0347) (Dako A/S)), 1:1000.

RESULTS

Calcium-induced Hydrophobicity and Purification of Native Grancalcin—One aim of this study was to determine if native grancalcin has a lipid anchor or other posttranslational modifications. Furthermore, we wanted to verify the reported N terminus of grancalcin. Since many EF-hand proteins expose hydrophobic areas upon binding of Ca$^{2+}$, we tested if grancalcin also becomes hydrophobic in the presence of high concentrations of Ca$^{2+}$. Human neutrophil cytosol was passed through a phenyl-Sepharose column in the presence of 0.5 mM Ca$^{2+}$, and the column was washed with Ca$^{2+}$-buffer. Replacement of Ca$^{2+}$ by an excess of EGTA led to elution of a limited number of proteins (Fig. 1A). By Coomassie staining, several bands can be seen, which all are expected to be Ca$^{2+}$-binding proteins or proteins that interact with Ca$^{2+}$-binding proteins. Indeed, several well known Ca$^{2+}$-binding proteins could be identified (data not shown). The band at 28 kDa, showing retarded EGTA elution, was identified as grancalcin by immunoblotting. Fractions containing grancalcin were further purified by anion-exchange chromatography as described under "Experimental Procedures." This resulted in pure grancalcin as evaluated by SDS-PAGE and immunoblotting (Fig. 1B). Thus, we here present a novel and fast protocol for purification of grancalcin from the cytosol of human neutrophils, based on its Ca$^{2+}$-dependent binding to a hydrophobic matrix.

Purification of Recombinant Grancalcin and Three Deletion Mutants—Recombinant grancalcin was produced and purified as described (3). The grancalcin clone was checked by conventional sequencing techniques and found to be identical with the published (2) sequence (data not shown). Recombinant grancalcin is synthesized as a glutathione S-transferase fusion protein linked by a cleavage site for thrombin and cleaved from prograncalcin (we use the term grancalcin for the hypothetical protein corresponding to the coding sequence, including the N-terminal Met) by an additional Gly-Ser dipeptide at the N terminus, as was confirmed by N-terminal sequence analysis (data not shown). The molecular mass of purified recombinant grancalcin was determined to be 24,154 Da, in good agreement with the theoretical mass of 24,154 Da.
in order to solve solubility problems (see below), we also generated three mutants with varying deletions in the N terminus, named after the starting amino acid according to the nomenclature for prograncalcin: ΔA2-grancalcin, ΔA50-grancalcin, and ΔA53-grancalcin, respectively. All three mutants were easily expressed and purified to electrophoretic homogeneity (Fig. 2) following the same procedure as for recombinant grancalcin. Our polyclonal (rabbit) anti-grancalcin antibodies reacted with all three mutants (data not shown).

**Peptide and Oligonucleotide Sequencing and Mass Spectrometry of Wild-type and Recombinant Grancalcin**—The purified wild-type grancalcin gave no signal in protein sequence analysis, indicating that the N terminus is blocked. Moreover, by mass spectrometry wild-type grancalcin was found to have a molecular mass of 23,856 Da as compared with 24,010 Da calculated for prograncalcin (hypothetical protein, corresponding to the coding sequence), suggesting that the N terminus of wild-type grancalcin is modified. Cleavage of wild-type grancalcin with endoproteinase Glu-C resulted in a number of fragments, one of which had a measured molecular mass (2829.4 Da, obtained in the negative mode) matching that of fragment 2–28 of prograncalcin if it is N-terminally acetylated (calculated 2828.2 Da). To confirm that this peptide was indeed the N-terminal fragment, it was further digested with thermolysin, resulting in two fragments with molecular masses of 1101.2 and 1746.8 Da, respectively. Sequence analysis of the latter showed the first 6 residues to be Phe-Ser-Ile-Gln-Val-Pro. This sequence and the molecular mass indicate that this fragment represents prograncalcin 12–28 (calculated 1745.1 Da). The molecular mass of the former fragment (obtained in the negative mode, using internal calibration) fits precisely to the cDNA-deduced sequence of prograncalcin 2–12 bearing an N-terminal acetyl group (1101.1 Da). Including this modification, the theoretical molecular mass of wild-type grancalcin becomes 23916 Da.

**Dimerization**—It was originally found that purified grancalcin exists as a homodimer (1), as also confirmed by the crystal structure (11). We wanted to examine if also cytosolic grancalcin and N-terminal mutants existed as dimers. Molecular sieve chromatography studies gave an apparent molecular mass of 40 kDa for recombinant grancalcin (Fig. 3A), and when cytosol was subjected to the same procedure and grancalcin was identified by immunoblotting, grancalcin also eluted as a 40-kDa protein. When the chromatography was performed in the presence of calcium, grancalcin eluted slightly later, with an apparent mass of 36 kDa. Thus, grancalcin is a dimer under all conditions, but the Ca2+-loaded form is slightly smaller, indicating conformational changes that make the complex more compact. N-terminal mutants all ran as dimers on molecular sieve chromatography (data only shown for ΔA2-grancalcin), verifying that the N terminus does not play a role in dimer formation. By chemical cross-linking with DSG, we could also verify that grancalcin is a functional dimer under all conditions tested (Fig. 3B) (data only shown for recombinant grancalcin and ΔA53-grancalcin).

**Ca2+-dependent Precipitation of Grancalcin and N-terminal Mutants**—Precipitation of grancalcin was dependent on both the concentration of protein and Ca2+, and the presence of detergent increased the threshold for Ca2+-induced precipitation (Fig. 4A). Therefore, we have performed subsequent experiments at low protein concentrations and repeated the experiments in the presence of detergent. Furthermore, because the first 52 amino acids are disordered in the crystals of grancalcin (11) and contain many hydrophobic residues that might be responsible for the pronounced precipitation, we tested N-terminal deletion mutants. The precipitation experiment of Fig. 4B illustrates that, as predicted, the N-terminal mutants are more soluble in the presence of Ca2+ than the full-length protein (only data for ΔA2- and ΔA53-grancalcin are shown).

**Direct Cation-binding Studies**—Evaluation of Ca2+ binding to recombinant grancalcin and N-terminal mutants by flow dialysis (Fig. 5) revealed two Ca2+-binding sites per monomer. In the absence of detergent (Fig. 5A), both recombinant grancalcin and the N-terminal mutants precipitated, but the N-terminal mutants precipitated only at the very end of the titration. In the presence of the detergent OG (Fig. 5B), the N-terminal mutants did not precipitate at all, whereas recombinant grancalcin precipitated at the very end. In the absence of detergent, recombinant grancalcin displayed a [Ca2+]50 value of 83 μM and moderate positive cooperativity (nH = 1.18). The truncated forms displayed [Ca2+]50 values of 140 μM (for ΔA2- and ΔA50-grancalcin) and 174 μM (for ΔA53-grancalcin) with no cooperativity (nH = 1.0). In the presence of 25 mM OG, recombinant grancalcin has a [Ca2+]50 of 25 μM with an nH of 1.1, whereas the truncated forms displayed a [Ca2+]50 of 35 μM (for ΔA2-grancalcin) to 50 μM (for ΔA50- and ΔA53-grancalcin) without any cooperativity or even slight negative cooperativity for ΔA53-grancalcin (nH = 0.94). Protein precipitation is a confounding factor in these flow dialyses, and the presence of detergent increases the affinity by a factor of 3, but it seems safe to conclude that grancalcin shows optimal Ca2+ sensitivity in the 25–85 μM range. The presence of 2 mM Mg2+ did not influence the binding curve (data not shown), indicating that the two sites are of the so-called Ca2+-specific type. Ca2+-specific sites seem to be common in the other proteins of the PE family, as was also reported for ALG-2 (12). The selectivity of the calpain light chain EF-hands is not known, but the affinity of the recombinant protein for Ca2+ corresponds to [Ca2+]50 values of 60–150 μM (27), thus similar to that of grancalcin. ALG-2 has two Ca2+-binding sites of high affinity around 1–3 μM and one site of low affinity of 300 μM (28).

**Intrinsic Trp Fluorescence**—Grancalcin contains two Trp residues (Trp118 and Trp124) as useful probes to monitor differences between wild-type and recombinant grancalcin as well as structural changes upon binding of Ca2+. After excitation at 278 nm of a solution of 1 μM wild-type or recombinant grancalcin in buffer A plus 37.5 mM OG, the emission fluorescence spectra of metal-free and Ca2+-saturated proteins show maxima between 325 and 335 nm (Fig. 6). This is characteristic for the emission of several Trp residues in a hydrophobic environment and was confirmed by the ~3-fold intensity decrease and 20-nm red shift of the fluorescence maximum when guanidine HCl was added. Saturating Ca2+ concentrations moderately increased the fluorescence, but Mg2+ had no influence (data not shown). It should be noted that all of the spectra of wild-type
and recombinant grancalcin are very similar, suggesting that their secondary structure and hydrophobic cores must be very similar. Moreover, the signal change of the metal-free proteins as a function of the concentration of guanidine HCl yielded $[\text{Gua-HCl}]_{0.5}$ values of 1.4 and 1.5 M for wild-type and recombinant protein (data not shown), indicating that both are equally stable.

**Binding Partner of Grancalcin**—In order to identify proteins that might interact with grancalcin, we made three affinity columns: one containing recombinant grancalcin in the presence of Ca$^{2+}$, one containing recombinant grancalcin without calcium, and one column with just the matrix as a negative control. At first, we applied a whole cell homogenate of human neutrophils to the columns in the presence of Ca$^{2+}$ and eluted with EGTA, but this did not result in any visible protein. However, when we applied the homogenate to the columns in the presence of EGTA, washed extensively, and then eluted with Ca$^{2+}$, a band of $67 \text{kDa}$ was found in both the eluate from the Ca$^{2+}$ (Fig. 7) and EGTA column but not from the mock column (not shown). The 67-kDa protein was concentrated by SDS-PAGE, cut out of the gel, and digested with trypsin, and the mixture of fragments was analyzed by mass spectroscopy. A data base search with this set of molecular masses identified L-plastin with a high score, covering 37% of the sequence distributed among 19 peptides with molecular masses matching the theoretical values within 50 ppm. L-plastin belongs to the plastin group of proteins, and L stands for leukocyte, indicating that it is the isotype present in leukocytes. Plastins are EF-hand proteins with actin-binding motifs, and they are known to have actin bundling activities. L-plastin has a molecular mass of 67 kDa and is well described in human neutrophils (26, 29, 30).

**Immunoprecipitation**—We substantiated the interaction between grancalcin and L-plastin by co-immunoprecipitation experiments followed by visualization by immunoblotting with anti-grancalcin and anti-L-plastin antibodies. When evaluating the immunoblots, it is important to remember that grancalcin will precipitate in the presence of Ca$^{2+}$ (as shown above), and this might introduce an artifact in the immunoprecipitation experiments. Immunoprecipitation of isolated cytosol from human neutrophils with L-plastin antibodies
was, in the absence of Ca$^{2+}$, able to also pull down grancalcin and vice versa as visualized by immunoblotting (Fig. 8, 1VI and 3VI). On the contrary, L-plastin could not be detected in the pellet, when cytosol was immunoprecipitated with anti-grancalcin antibodies in the presence of Ca$^{2+}$ (Fig. 8, 3III). We therefore suggest that the fact that grancalcin could be found in the anti-L-plastin pellet is due to precipitation rather than immunoprecipitation. The Ca$^{2+}$ specificity of the interaction between grancalcin and L-plastin is further supported by the fact that the L-plastin antibody LPL7,2, which only recognizes the Ca$^{2+}$-loaded form of L-plastin, was not able to pull down grancalcin in the absence of Ca$^{2+}$ (Fig. 8, 2VI). As a negative control, we also probed the immoprecipitates by immunoblotting with anti-MRP14 antibodies. MRP14 is a 14-kDa EF-hand protein that is highly enriched in the cytosol of human neutrophils (31). We did not detect any immunoprecipitation of MRP14 with either anti-grancalcin (Fig. 8, 4III and 4VI) or anti-L-plastin antibodies (data not shown). The specific co-immunoprecipitation of grancalcin and L-plastin in the absence of Ca$^{2+}$ further corroborates the interaction between the two proteins.

**DISCUSSION**

**Purification and N Terminus**—The purpose of this study was to provide a detailed molecular description of the interaction of grancalcin with Ca$^{2+}$ and the ensuing conformational changes and to identify a possible binding partner to grancalcin. The Ca$^{2+}$-dependent binding of grancalcin to a phenyl-Sepharose column was used as a very efficient first step in the purification of wild-type grancalcin from the cytosol of human neutrophils. In this single chromatographic step, a limited number of proteins, all expected to be Ca$^{2+}$-binding proteins, were highly enriched. Immunoblotting showed grancalcin to be present, and wild-type grancalcin could be separated from the other proteins in the eluate by subsequent ion exchange chromatography.

Sequence analysis of pure wild-type grancalcin suggested that the N-terminal amino group is blocked. The molecular mass of wild-type grancalcin is in agreement with posttranslational removal of the N-terminal methionine and acetylation of
formational changes upon binding of Ca\(^{2+}\) and interact in a Ca\(^{2+}\) saturated form is partly insoluble, depending on the protein concentration. Exposure of hydrophobic residues is probably the reason, since the protein is much more soluble in nonionic detergents and binds in a Ca\(^{2+}\)-dependent way to phenyl-Sepharose. Grancalcin is much more soluble in the presence of Ca\(^{2+}\) than ALG-2 (12), and with working concentrations of grancalcin below 2 \(\mu\)M, Ca\(^{2+}\) precipitation does not seem to occur. Several EF-hand proteins bind to phenyl-Sepharose upon binding of Ca\(^{2+}\), but it is unusual for them to aggregate. It may be typical for the PEF subfamily, since ALG-2 (12, 13) and calpain (34) also form insoluble aggregates upon binding of Ca\(^{2+}\). The PEF proteins all have hydrophobic N termini, which could be of importance for precipitation. We therefore decided to generate N-terminal deletion mutants in order to test if this would improve the solubility in the presence of Ca\(^{2+}\). Indeed, we found that all three mutants were more soluble than the intact protein. We conclude that the N terminus is of importance for Ca\(^{2+}\)-mediated precipitation. This suggests that conformational changes upon binding of Ca\(^{2+}\) are transduced into the N terminus to give important altered tertiary structure. Unfortunately, the crystal structure of grancalcin cannot help us in this regard, because the N terminus was disordered (11).

**Ca\(^{2+}\) Binding**—Comparison of the sequence of EF-hands in grancalcin with those in well known Ca\(^{2+}\)-binding proteins (6) led to the following predictions about their capacity to bind Ca\(^{2+}\): EF1 is very similar (64% identity) to that of calpain dVI and should bind Ca\(^{2+}\); EF2 is abortive, since the \(Z\) position is occupied by Ala instead of Glu; EF3 is a canonical EF-hand with the correct residues for the coordination of Ca\(^{2+}\); EF4 is completely abortive due to lack of oxygen-carrying residues in three critical Ca\(^{2+}\)-coordinating positions; and EF5 is very similar to that of calpain dVI (i.e., an abortive Ca\(^{2+}\)-binding site) but well suited for dimerization. Flow dialysis confirmed the prediction that in grancalcin only two EF-hands, likely to be EF1 and EF3, bind Ca\(^{2+}\) with moderate affinity (\(1\) [(Ca\(^{2+}\)]\(_{50}\) = 25–83 \(\mu\)M) and positive cooperativity (\(n_{H} = 1.18\)). The crystalization of apogranalcin revealed that EF1 is not in a conformation that allows for Ca\(^{2+}\) binding, since the X and Y liganding oxygens are directed away from the center of the loop (11). EF3 displays a Ca\(^{2+}\)-binding conformation, but even in the “calcium grancalcin” crystal only one Ca\(^{2+}\) ion was bound to one EF3 in the dimer. The corresponding site in the second molecule of the dimer is unoccupied and shows a much higher mobility. The conformations of apogranalcin and grancalcin with one Ca\(^{2+}\) are very similar. Moreover, the EF3-bound Ca\(^{2+}\) still seems to have a high mobility. Thus, the following binding model can be postulated. The first Ca\(^{2+}\) binds to one EF3 hand with rather low affinity and without provoking substantial conformational changes. Then the second EF3 binds Ca\(^{2+}\), leading to a reorientation of the hydroxyl group of Ser\(^{136}\), which supposes a major conformational change. The latter may activate EF1 in each subunit to bind Ca\(^{2+}\). Once activated, the affinity of EF1 may be higher than that of EF3, thus explaining the positive cooperativity. The forms with the truncated N-terminal domain still bind two Ca\(^{2+}\) ions/monomer but with an approximate 2-fold lower affinity and absence of positive cooperativity, suggesting that this domain is needed for cross-talk between EF1 and EF3 in fully Ca\(^{2+}\)-saturated grancalcin. Since the structure of grancalcin with more than one bound Ca\(^{2+}\) could not be resolved due to precipitation of the protein (11), we expect that structural studies on the more soluble N-terminal mutants will allow the proposed binding model to be tested. The moderate affinity of grancalcin for Ca\(^{2+}\) is within the physiological range, especially at locations near the plasma membrane or near internal stores where the local Ca\(^{2+}\) changes can exceed 500 \(\mu\)M in activated human neutrophils (35). Furthermore, it is likely that the Ca\(^{2+}\) affinity for grancalcin increases in a hydrophobic environment as indicated by the prolonged elution of grancalcin from the hydrophobic column as compared with other Ca\(^{2+}\)-binding proteins and by the higher Ca\(^{2+}\)-affinity in the presence of detergent. Enhanced Ca\(^{2+}\) affinity when bound to membranes has been shown for other Ca\(^{2+}\)-binding proteins (e.g. protein kinase C (36)).

**Binding Partner**—We found that L-plastin interacts with grancalcin and that Ca\(^{2+}\) regulates the interaction in a negative fashion. L-plastin, like other plastin isoforms and fimbrin, is a mosaic protein containing 2 EF-hands, a calmodulin-binding domain, and two actin-binding domains (37). In resting cells, the Ca\(^{2+}\)-binding sites are unoccupied, and most of L-plastin is involved in the cross-linking of F-actin fibers. Following stimulation of leukocytes with inflammatory stimuli such as formyl-methionine-leucine-phenylalanine or immune complexes that bind to Fcy receptors, L-plastin is phosphorylated at Ser\(^{5}\), and this in turn leads to integrin activation and subsequent increased adhesion (26, 29). At present, it is not known if the L-plastin-grancalcin complex in the absence of Ca\(^{2+}\) still has actin-bundling activity and if, in this complex, Ser\(^{5}\) can still...
be phosphorylated. Cell stimulation leads to a cytoplasmic Ca\(^{2+}\) rise with two consequences for L-plastin: 1) its actin-bundling activity is inhibited, at least in \textit{in vitro} experiments (26); and 2) the L-plastin-grancalcin complex dissociates (this study). Thus, Ca\(^{2+}\) regulates the activity of L-plastin in a complex way, via endogenous EF-hand motifs and via grancalcin. At present it is not clear if binding of Ca\(^{2+}\) to L-plastin, to both, is necessary to inhibit the interaction between the two proteins. It is intriguing that calpain, the close relative of grancalcin, or to both, is necessary to inhibit the interaction as well as the structural aspects of this interaction. Further experiments will hopefully clarify the functional role of grancalcin and the importance of the interaction with L-plastin as well as the structural aspects of this interaction.

Acknowledgments—We thank Hanne Kristensen and Allan Kastrup for technical assistance and Jack B. Cowl and Kim Theilig-Mønch, Daniel J. Carter, and Ole E. Sørensen for comments on the manuscript.

REFERENCES
1. Teahan, C. G., Totty, N. F., and Segal, A. W. (1992) \textit{Biochem. J.} \textbf{286}, 549–554
2. Boyhan, A., Casimir, C. M., French, J. K., Teahan, C. G., and Segal, A. W. (1992) \textit{J. Biol. Chem.} \textbf{267}, 2928–2933
3. Løllike, K., Sørensen, O., Bundgaard, J. R., Segal, A. W., Boyhan, A., and Borregaard, N. (1995) \textit{J. Immunol. Methods} \textbf{185}, 1–8
4. Maki, M., Narayana, S. V. L., and Hitomi, K. (1997) \textit{Biochem. J.} 718–720
5. Kitaura, Y., Watanabe, M., Satoh, H., Kawai, T., Hitomi, K., and Maki, M. (1999) \textit{Biochem. Biophys. Res. Commun.} \textbf{263}, 68–75
6. Nakayama, S., and Kreitsinger, R. H. (1994) \textit{Annu. Rev. Biophys. Biomol. Struct.} \textbf{23}, 473–507
7. Lin, G., Chattopadhyay, D., Maki, M., Wang, K. K. W., Carson, M., Jin, L., Yuen, P., Takano, E., Hatanaka, M., DeLucas, L. J., and Narayana, S. V. L. (1997) \textit{Nat. Struct. Bio.} \textbf{4}, 539–547
8. Blanchard, H., Grochułski, P., Li, Y., Arthur, S. C., Davis, P. L., Elce, J. S., and Cygler, M. (1997) \textit{Nat. Struct. Bio.} \textbf{4}, 532–538
9. Yoshizawa, T., Soriuchi, H., Tomioka, S., Ishiura, S., and Suzuki, K. (1995) \textit{Biochem. Biophys. Res. Commun.} \textbf{208}, 376–383
10. Han, Q., Jia, J., Li, Y., Lollike, K., and Cygler, M. (2000) \textit{Acta Crystallogr. Sec. D} \textbf{56}, 772–774
11. Jia, J., Han, Q., Borregaard, N., Lollike, K., and Cygler, M. (2000) \textit{J. Mol. Biol.} \textbf{300}, 1271–1281
12. Maki, M., Yamauchi, K., Kitaura, Y., Sadah, H., and Hitomi, K. (1998) \textit{J. Biochem.} \textbf{124}, 1170–1177
13. Lo, K. W. H., Zhang, Q., Li, M., and Zhang, M. (1999) \textit{Biochemistry} \textbf{38}, 7498–7508
14. Borregaard, N., Kjeldsen, L., Lollike, K., and Sengeløv, H. (1992) \textit{FEBS Lett.} \textbf{304}, 195–197
15. Takeyama, Y., Nakizishir, H., Urataku, Y., Nishimoto, A., and Nishizuka, Y. (1986) \textit{FEBS Lett.} \textbf{194}, 110–114
16. Winacy, L., and Kuznicki, J. (1995) \textit{J. Neurochem.} \textbf{65}, 381–388
17. Zuzulya, S., and Stryler, L. (1992) \textit{Proc. Natl. Acad. Sci. U. S. A.} \textbf{89}, 11569–11573
18. Cox, J. A. (1996) in \textit{Guidebook to the calcium-binding Proteins} (Celio, M. R., Pauls, T., and Schwaller, B., eds) pp. 1–12, Oxford University Press, Oxford
19. Boyum, A. (1968) \textit{Scand. J. Clin. Lab. Invest.} \textbf{21}, 77–90
20. Laemml, U. K. (1970) \textit{Nature} \textbf{227}, 680–685
21. Towbin, H., Staehelin, T., and Gordon, J. (1979) \textit{Proc. Natl. Acad. Sci. U. S. A.} \textbf{76}, 4350–4354
22. Borregaard, N., Heiple, J. M., Simons, E. R., and Clark, R. A. (1983) \textit{J. Cell Biol.} \textbf{97}, 52–61
23. Colowick, S. P., and Womack, F. C. (1969) \textit{J. Biol. Chem.} \textbf{244}, 774–777
24. Wilm, M., Shevchenko, A., Houthaeve, T., Breit, S., Schweigerer, L., Potis, T., and Mann, M. (1996) \textit{Nature} \textbf{379}, 466–469
25. Appe, R. D., Bairoch, A., and Hochstrasser, D. P. (1994) \textit{Trends Biochem. Sci.} \textbf{19}, 258–260
26. Jones, S. L., and Brown, E. J. (1996) \textit{J. Biol. Chem.} \textbf{271}, 14623–14630
27. Minami, Y., Enori, Y., Imajo-Ohmi, S., Kawasaki, K., and Suzuki, K. (1988) \textit{J. Biochem. (Tokyo)} \textbf{104}, 927–933
28. Tarabyskina, S., Møller, A. L., Durussel, I., Cox, J. A., and Berchtold, M. W. (2000) \textit{J. Biol. Chem.} \textbf{275}, 10514–10518
29. Jones, S. L., Wang, J., Turek, C. W., and Brown, E. J. (1998) \textit{Proc. Natl. Acad. Sci. U. S. A.} \textbf{95}, 9331–9336
30. Wang, J., and Brown, E. J. (1999) \textit{J. Biol. Chem.} \textbf{274}, 24349–24356
31. Hessian, P. A., Edgeworth, J., and Hogg, N. (1993) \textit{J. Leukoc. Biol.} \textbf{53}, 197–205
32. Bradshaw, R. A., Brickey, W. W., and Walker, K. W. (1998) \textit{Trends Biochem. Sci.} \textbf{23}, 263–267
33. Creighton, T. E. (1993) in \textit{Proteins} (Creighton, T. E., ed) pp. 49–109, W. H. Freeman and Co., New York
34. Dutt, P., Arthur, J. S. C., Cron, D. E., and Elce, J. S. (1998) \textit{FEBS Lett.} \textbf{436}, 367–371
35. Pettit, E. J., Davies, E. V., and Hallett, M. B. (1997) \textit{Histol. Histopathol.} \textbf{12}, 479–490
36. Mosier, M., and Eppard, R. M. (1997) \textit{Mol. Membr. Bio.} \textbf{14}, 65–70
37. Zuz, Y., Kohno, M., Kubota, I., Nishida, E., Hanaoka, M., and Namba, Y. (1999) \textit{Biochemistry} \textbf{28}, 1055–1062
38. Hutenlocher, A., Palecek, P. S., Lu, Q., Zhang, W., Mellgren, R. L., Laufenburger, D. A., Ginsberg, M. H., and Horwitz, A. F. (1997) \textit{J. Biol. Chem.} \textbf{272}, 32719–32722
39. Albeida, S. M., and Buck, C. A. (1999) \textit{FASEB. J.} \textbf{4}, 2868–2880