Identification and Characterization of a Calmodulin-binding Domain in Ral-A, a Ras-related GTP-binding Protein Purified from Human Erythrocyte Membrane*

(Received for publication, December 4, 1996, and in revised form, April 11, 1997)

Kai Ling Wang‡, M. Tariq Khan§, and Basil D. Roufogalis¶

From the Department of Pharmacy, University of Sydney, New South Wales 2006, Australia

© 1997 by The American Society for Biochemistry and Molecular Biology, Inc.

A 28-kDa protein (p28) has been purified from Triton X-100 extracts of human erythrocyte plasma membrane by calmodulin affinity chromatography. Based on internal peptide sequencing and its protein amino acid composition, this protein has been shown to be highly related, if not identical to, Ral-A, a Ras-related GTP-binding protein. This protein assignment is consistent with the findings that p28 binds[^32P]GTP specifically and has low GTPase activity. In this study we describe the identification and characterization of a calmodulin-binding domain in Ral-A. The Ca^{2+}-dependent interaction of p28 with calmodulin was first detected by a calmodulin affinity column. Gel overlay experiments of both p28 and recombinant Ral-A with biotinylated calmodulin affinity column. Gel overlay experiments of both p28 and recombinant Ral-A with biotinylated calmodulin were defrayed in part by the payment of page charges. This article must be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

A15, University of Sydney, NSW, 2006, Australia. Tel.: 61-2-9351-3234; Fax: 61-2-9351-4447; E-mail: basir@pharm.usyd.edu.au.

The abbreviations used are: GDS, GDP dissociation stimulator; PAGE, polyacrylamide gel electrophoresis; CAPS, 3-(cyclohexylamino)propanesulfonic acid; PVDF, polyvinylidene difluoride; GAP, GTPase activating protein; HPLC, high pressure liquid chromatography.

The Ras superfamily of low molecular mass (20–29 kDa) GTP-binding proteins is involved in the regulation of a wide variety of cellular functions and in signal transduction (1–3). Functioning as a molecular switch the protein transduces signals in the active GTP-bound form and is converted to an inactive form when the bound GTP is hydrolyzed to GDP. Its intrinsic GTPase activity is regulated by guanine nucleotide exchange factors such as GDP dissociation inhibitor (GDI); GDP dissociation inhibitor, and GTPase activating protein (GAP).

Ral proteins represent a distinct family of Ras-related GTP-binding proteins. They share more than 50% amino acid sequence identity with Ras, their nucleotide binding and GTP hydrolysis activities being comparable with those of Ras. The Ras genes were originally isolated from a cDNA library of immortalized simian B-lymphocytes (4). The isolation of additional Ras cDNAs from human pheochromocytoma and HL-60 leukemia libraries has revealed the existence of two forms of the gene, ras-A and ras-B, which are about 85% identical but differ essentially in their C-terminal region (5). Ral gene products are expressed in human platelet membrane (6), as well as in most cell types, with particularly high levels in brain and testis (7, 8). Ral proteins have a diverse subcellular localization, not only in plasma membrane, but also in cytoplasmic vesicles, including clathrin-coated vesicles and secretory vesicles (9, 10). Like other members of the Ras superfamily, Ral proteins have their own set of highly specific regulatory factors. RalGAP has been identified and characterized from the cytosolic fraction of rat brain and mouse testis (11) as well as human platelet (12). RalGDS was found to interact with ras p21 and to function as a putative effector protein in Ras signaling pathways (13–15). Recently, several groups have reported that Ral proteins and RalGDS constitute a distinct downstream pathway from Ras that can induce cellular transformation in parallel with activation of the Raf/mitogen-activated protein kinase cascade (16–18). This signaling pathway from Ras to Ral through RalGDS is selectively regulated by Rap1 (19, 20). In addition, the effector protein of Ral, RLIP (Ral interacting protein), has been shown to contain a GAP region related to RhoGAP domains and to have GAP activity acting upon CDC42 and Rac (21). Moreover, Ral-A has been documented to be involved in the tyrosine kinase-mediated activation of phospholipase D, suggesting that the signaling pathway from Ras to Ral leads to the regulation of phospholipid metabolism (22). Ral proteins are biochemically well characterized GTPases. However, little is known about their physiological functions, because no biological activity associated with its overexpression in cells has been detected (11).

In this study, we present evidence that a monomeric GTP-binding protein with molecular mass of 28 kDa (p28) purified from human erythrocyte membranes is highly related, if not identical to, Ral-A, as determined by internal peptide sequencing and amino acid composition analysis. This has also been confirmed by the measurement of its GTP binding and hydrolysis. The data show for the first time that Ral-A is a calmodulin-binding protein. A putative calmodulin-binding domain in Ral-A was identified in its C-terminal region based on its sequence identity with Ras, raising the possibility that it may be associated with Ca^{2+}-dependent intracellular signaling pathways.
Calmodulin has been characterized. Very recently, Fischer et al. have reported the presence of a calmodulin-binding domain in the C-terminal region of Gem/Kir, a subfamily of Ras-related GTP-binding proteins (23). Rin, a neuron-specific, Ras-related GTP-binding protein, has been also shown to bind calmodulin through a C-terminal binding motif (24). The present study identifying a calmodulin-binding domain in RaI supports these two findings and in addition shows the calmodulin binding domain to be present in a Ras-related protein containing the CAAX motif, lacking in the previously identified examples. Work is currently in progress to investigate the function of this calmodulin-binding domain in RaI. Preliminary reports of this work have appeared previously in a conference proceeding (25).

**EXPERIMENTAL PROCEDURES**

**Materials**—Packed red blood cells were obtained from the Red Cross blood bank, Sydney (NSW, Australia). [α-32P]GTP (3000 Ci/mmol) and [14C]formaldehyde (40–60 mCi/mmol) were purchased from DuPont NEN. Calmodulin-agarose, ATP, GTP, 5′-adenyllylimidodiphosphate, avian alkaline phosphatase, nitro blue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate, and the reagents for SDS-PAGE were from Sigma. Calmodulin and biotinylated calmodulin were obtained from Calbiochem. PVDF membranes were from Bio-Rad. An 18-aminoc acid peptide (P18) with the sequence of SEKKNKGGKSSLAKR was synthesized by Chiron Mimotopes Pty. Ltd. (VIC, Australia). Other materials and chemicals were the highest grade available from commercial sources. Recombinant RaI was a generous gift from Drs. Hiroshi Koide and Yoshito Kaziro at the Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, Japan.

**Protein Purification**—p28 protein was purified from human red blood cell membrane as described previously (26), with the following modifications. Briefly, calmodulin-depleted human plasma membranes were prepared according to the method of Wang et al. (27). The membranes (0.5–1.0 g of protein) were solubilized for 60 min at 4 °C in 200 mM KCl, 1 mM MgCl2, 200 μM CaCl2, 20 mM HEPES, pH 7.4, 0.55% (w/v) Triton X-100, and 20% (v/v) glycerol. After centrifugation at 50,000 × g for 30 min, the supernatant collected from the solubilisate was applied to a calmodulin-agarose column (10 × 1.5 cm) pre-equilibrated with the solubilisation buffer. The column was washed rapidly with 500 ml (about 50 column volumes) of washing buffer (200 mM KCl, 20 mM HEPES, pH 7.4, 0.1% (w/v) Triton X-100, and 20% (v/v) glycerol). After centrifugation at 50,000 × g for 30 min, the supernatant collected from the solubilisate was applied to a calmodulin-agarose column (10 × 1.5 cm) pre-equilibrated with the solubilization buffer. The column was washed rapidly with 500 ml (about 50 column volumes) of washing buffer (200 mM KCl, 20 mM HEPES, pH 7.4, 0.1% (w/v) Triton X-100, and 20% (v/v) glycerol). The fractions were collected and stored at –80 °C. For further purification, the obtained p28 were dialyzed against changes of 2 liters of elution buffer without KCl and EDTA to reduce the salt concentration, and then loaded onto a DEAE-cellulose column (5 × 1.5 cm) pre-equilibrated with equilibration buffer (10 mM KCl, 50 mM Tris-HCl, pH 7.4, 20% (v/v) glycerol). The column was washed with 100 ml of equilibration buffer. The protein was eluted with a buffer of 1 mM KCl, 50 mM Tris-HCl, pH 7.4, 20% (v/v) glycerol. The fractions were collected and analyzed by SDS-PAGE. The relative abundance of each amino acid (Asx, Gln, Glu, Gly, Thr, Ala, Pro, Trp, and Lys) was determined by measuring its concentration compared with an internal standard (125 pmol/amino acid; calibration kit, Sigma). However, during the hydrolysis process, tryptophan was destroyed by hydrochloric acid, and cysteine and lysine were oxidized. These three amino acids could not therefore be detected. Moreover, aspartic acid and asparagine as well as glutamic acid and glutamine were converted to one peak instead of separate peaks. Thus, asparagine and glutamine were converted to their corresponding acids during hydrolysis so that Asp and Asn (Asx) and Glu and Gln (Glx) were counted together. Glycine values are inaccurate due to contamination from the gel running buffer after protein samples are analyzed by SDS-PAGE and electrotransferred to PVDF membrane. A special search program (Constellation 4) in SWISS two-dimensional PAGE amino acid composition analysis (available through the ExPasy server at the University of Geneva, accessed via World Wide Web) has therefore been developed that omits glycine and uses the remaining 15 amino acids for comparison of composition values. The score in this program is calculated by computing the euclidian distance between the search protein amino acid composition and the amino acid composition of all proteins in the SWISS-PROT database.

**Guanine Nucleotide Binding**—p28 GTP-binding activities were detected by two assays: (i) Photofaffinity labeling of p28 with [α-32P]GTP was carried out using the method of Nakaoka et al. (29). The pure protein (about 1 μg) was incubated with 5 μCi of [α-32P]GTP (3000 Ci/mmol) in buffer containing 200 mM KCl, 20 mM HEPES, pH 7.4, 2.5 mM EDTA, 0.1% (w/v) Triton X-100, 20% (v/v) glycerol, 100 μM 5′-adenyllylimidodiphosphate, 5 mM MgCl2, placed in an ice bath, and irradiated with a UV lamp (Mineralight model UVGL-58, 254 nm) at a distance of 8 cm from 30 min. When the specific binding of [α-32P]GTP to p28 was studied, the sample was preincubated with competing substrate GTP (100 μM) at room temperature for 10 min. After irradiation, the samples were precipitated with 10% trichloroacetic acid and subjected to 6–14% SDS-PAGE and then transferred to PVDF membrane. The PVDF membranes were air dried and exposed to a Storage Phosphor Screen for 72 h. The [α-32P]GTP-binding proteins were detected by autoradiography with a Phosphor Imager (Molecular Dynamics). (ii) [α-32P]GTP binding was followed after electrophoretting onto PVDF membranes. For the detection of [α-32P]GTP-binding proteins on PVDF membranes, the modified procedure of Bhulk et al. and Haslam et al. (30) was followed. The PVDF membranes were first washed with 50 mM Tris-HCl, pH 7.4, containing 2 mM MgCl2 and 0.3% Tween 20, and then incubated for 30 min at room temperature in the same buffer containing 1 μCi [α-32P]GTP (3000 Ci/mmol). The PVDF membranes were washed extensively with the same buffer, air dried, and then exposed to a Storage Phosphor Screen (Molecular Dynamics) for 72 h. The [α-32P]GTP binding proteins were revealed by autoradiography on a Phosphor Imager (Molecular Dynamics).

**Biotinylated Calmodulin Overaly**—About 1–2 μg of purified p28 and recombinant RaI were subjected to 12% SDS-PAGE and electropherotically transferred to PVDF membranes. The PVDF membranes containing p28 or recombinant RaI were incubated for 3 h with blocking buffer (5% (w/v) powdered skim milk, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.2% (w/v) Tween 20) with gentle shaking at room temperature and washed three times with the same buffer. The membranes were then incubated with blocking buffer containing 80 ng/ml of biotinylated calmodulin and 1 mM CaCl2 for 2 h with continuous shaking at room temperature. In the control, 1 mM CaCl2 was replaced by 5 mM EGTA in the above steps. After three further washes with blocking buffer for 5 min each, the membranes were incubated for 2 h with 1:1000 diluted avidin–alkaline phosphatase (Sigma) in blocking buffer at room temperature and then washed three times with blocking buffer and finally with substrate buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 50 mM MgCl2). The color development was achieved by incubating the membranes in a reaction mixture containing 0.01% nitro blue tetrazolium and 0.05% 5-bromo-4-chloro-3-indolyl phosphate in 10 ml of substrate buffer. The reaction was allowed to proceed at room temperature until the color change was complete. The PVDF membrane-bound p28 and recombinant RaI were also visualized by staining with 0.1% (w/v) Amido Black, 40% (v/v) methanol, and 10% (v/v) acetic acid and destaining with 40% (v/v) methanol and 10% (v/v) acetic acid.

**Identification of a Putative Calmodulin-binding Domain in RaI—**
Computer analysis of Ral-A sequence was carried out by Dr. Jose Martin-Nieto (Instituto de Investigaciones Biomédicas, Madrid, Spain). PLOT.A/GGR and PLOT.A/G2R computer programs assign the predicted secondary structure for each residue in a protein sequence and provide approximate boundaries for the helical region. PLOT.A/HEL program draws a putative helical stretch by projecting the positions of the amino acids onto a plane perpendicular to the helix axis.

To quantitate the information presented on the helical wheel projection, the mean hydrophobic moment for this sequence was calculated according to the method of Erickson-Viitanen and DeGrado (31) using the hydrophobicity scales of Eisenberg et al. (32).

\[
\langle \mu \rangle = \left[ \left( \sum_{i=1}^{n} H_b \sin (100^\circ \times n) \right)^2 + \sum_{i=1}^{n} H_b \cos (100^\circ \times n) \sum_{i=1}^{n} H_b \right]^{1/2} / n
\]

(Eq. 1)

\( H_b \) is the hydrophobicity of the \( i \)th amino acid, and \( n \) is the length of the sequence of interest. The term 100° corresponds to the angle at which successive side chains emerge from an \( \alpha \)-helix when viewed down the axis.

**Calmodulin Overlap with \[^{14}\text{C}]\text{P18.—}** A 18-amino acid peptide (P18) with the sequence SKENKKRRRLKRR corresponding to a putative calmodulin-binding domain in Ral-A was synthesized by Chiron Mimotopes Pty. Ltd. P18 was then radioactively labeled with \[^{14}\text{C}\text{fornaldehyde (DuPont NEN) by the method of Rice and Means (33). The specific radioactivity of \[^{14}\text{C}\text{labeled P18 was estimated to be 7.0 \times 10^6}\text{cpm/mg protein from a plot of scintillation counts/minute versus peptide concentration.} \]5 \mu \text{g of calmodulin was subjected to 6–16\% SDS-PAGE and amino acids were recovered to PVDF membranes. One blot was incubated with 100 \mu \text{m of \[^{14}\text{C}\text{P18 and 1 \text{mM CaCl}}_2\text{ in 10 ml of washing buffer (50 \text{mM Tris-HCl, pH 7.4, and 0.3\% (w/v) Tween 20); the other was incubated with 100 \mu \text{m of \[^{14}\text{C}\text{P18 and 1 \text{mM EDTA in 10 ml of washing buffer separately for 30 min at room temperature.} \]The blots were soaked in 20 ml of washing buffer and washed five times. The PVDF membrane-bound calmodulin was visualized by staining with 0.1\% (w/v) Amido Black, 40\% (w/v) methanol, and 10\% (w/v) acetic acid and destaining with 40\% (w/v) methanol and 10\% (w/v) acetic acid. Blots to be counted were sliced transversely into 2-mm strips. The slices were soaked in 5 ml of Econofluor overnight and then counted in a liquid scintillation analyzer (1900A, TRI-CARB, Packard Pty. Ltd.).**

**Competitive Binding Assay of P28 and P18 with a Calmodulin-Agarose Resin—** Constant amounts of p28 and calmodulin-agarose resin were used in all experimental procedures. Samples of p28 (about 1.5 \mu \text{g}) were incubated with 0.5 \mu \text{g of calmodulin-agarose resin in Eppendorf tubes containing 250 \text{mM KCl, 50 \text{mM Tris-HCl, pH 7.4, 20\% (w/v) glycerol, 0.05\% (w/v) Triton X-100, 1 \text{mM MgCl}}_2\text{, and 1 \text{mM CaCl}}_2\text{ in the presence and in the absence of 10 \mu \text{g of P18 or 10 \mu \text{g of calmodulin at 4 \text{°C overnight.} \]The samples were then centrifuged at 10,000 \times g in a microcentrifuge for 10 s, supernatants were removed, and proteins (referred to as supernatant) were subjected to SDS-PAGE analysis. The calmodulin-agarose resin samples were washed with 1.5 ml of washing buffer (200 \text{mM KCl, 50 \text{mM Tris-HCl, pH 7.4, 20\% (w/v) glycerol, and 0.05\% (w/v) Triton X-100}) at 4 \text{°C for 10 min.} \]The washing solutions were discarded after centrifugation at 10,000 \times g in a microcentrifuge for 10 s. This washing step was repeated five times. The calmodulin-agarose resin samples were then incubated with 1 ml of elution buffer (200 \text{mM KCl, 50 \text{mM Tris-HCl, pH 7.4, 20\% (w/v) glycerol, 0.05\% (w/v) Triton X-100, and 5 \text{mM EDTA, pH 7.4 at 4 \text{°C for 5 h.} \]After centrifugation at 10,000 \times g in a microcentrifuge for 10 s, the supernatants were removed, and proteins were recovered to PVDF membranes. One blot was incubated with 100 \mu \text{m of \[^{14}\text{C}\text{P18 and 1 \text{mM CaCl}}_2\text{ in 10 ml of washing buffer separately for 30 min at room temperature.} \]The blots were soaked in 20 ml of washing buffer and washed five times. The PVDF membrane-bound calmodulin was visualized by staining with 0.1\% (w/v) Amido Black, 40\% (w/v) methanol, and 10\% (w/v) acetic acid and destaining with 40\% (w/v) methanol and 10\% (w/v) acetic acid. Blots to be counted were sliced transversely into 2-mm strips. The slices were soaked in 5 ml of Econofluor overnight and then counted in a liquid scintillation analyzer (1900A, TRI-CARB, Packard Pty. Ltd.).**

**Internal Peptide Sequencing—** Eight tryptic peptides selected from the protein sample (p28) were separated by reversed phase HPLC and sequenced. One of them gave a sequence of VEDENVVPFLVGN at an initial yield of 13.5 pmol and a repetitive yield of 84.4\%. The other gave a sequence of AEQWNLV at an initial yield of 2.7 pmol and a repetitive yield of 79.7\%. A search of the SWISS-PROT data base identified the peptides as having originated from the 23.6-kDa Ras-related protein Ral-A (SWISS-PROT accession number P11233). The two peptides came from Ral-A sequence positions 114–127 and 146–150, respectively, and were consistent with expected tryptic digestion products. Based on the absolute homology of internal peptide sequence, p28 was identified as Ral-A.

**Protein Amino Acid Composition Analysis—** The p28 amino acid composition was used to search and obtain a score in the SWISS-PROT data base. The protein at the top of the closest SWISS-PROT entries in human species as well as the closest SWISS-PROT entries in cross-species was the Ras-related, GTP-binding protein, Ral-A. Ral-A was distinguished from other protein candidates by its significant differences in score. Moreover, the protein molecular mass (which is estimated to be 28 kDa from SDS-PAGE in Fig. 1) and pI value (which is calibrated to be 7.0 based on its amino acid composition) provided strong additional information to rule out incorrect candidates from the list. With these guidelines, it was concluded that Ral-A was the correct candidate for p28, because Ral-A appeared to be the highest match in both single and cross-species data base searches. Fig. 2 shows the amino acid composition comparison of p28 with Ral-A obtained from SWISS-PROT (accession number P11233).
**A Calmodulin-binding Domain in Ral-A**

**Guanine Nucleotide Binding—** p28 GTP-binding activities were detected by photoaffinity labeling and gel overlay with $[\alpha^32P]GTP$ (see Fig. 3). Panel A in Fig. 3 shows that p28 protein was photoaffinity labeled with $[\alpha^32P]GTP$. The specificity of this labeling was evident from the ability of unlabeled 0.1 mM GTP to prevent the covalent labeling. The p28 protein band on the autoradiogram, as well as on the PVDF membrane (not shown), appeared broader than normal on SDS-PAGE, but this could be due to p28 oxidation under irradiation. Increasing covalent labeling of p28 with $[\alpha^32P]GTP$ corresponded to increased irradiation time. Panel B in Fig. 3 shows that p28 can be specifically labeled by $[\alpha^32P]GTP$. The specificity of labeling was shown from the finding that the presence of competing substrate 0.1 mM GTP completely blocked p28 labeling with $[\alpha^32P]GTP$, whereas 0.1 mM ATP did not abolish GTP-binding. When overlaid, the band on the autoradiogram fell exactly on the Amido Black-stained band detected on the PVDF membrane. Low molecular mass GTP-binding proteins are known to renature after being electrophoretically transferred to PVDF membranes or nitrocellulose blots (unlike heterotrimeric G proteins) and exhibit specific binding to guanine nucleotides following incubation of the blots with $[\alpha^32P]GTP$ (30, 36). Our results suggest that a renatured form of p28 on PVDF membrane retains the ability to specifically bind to GTP. It was therefore concluded that p28 is a low molecular mass GTP-binding protein, consistent with its identification as Ral-A or a highly related Ral-A protein.

**Biotinylated Calmodulin Overlay—** Using biotin as a reporter ligand to detect biotinylated calmodulin bound to renatured calmodulin-binding proteins (34), biotinylated calmodulin overlay showed binding of calmodulin to purified p28 (Fig. 4, lane 1). No binding of biotinylated calmodulin was observed when 5 mM EGTA replaced 1 mM CaCl$_2$ (Fig. 4, lane 2), indicating that the interaction of biotinylated calmodulin with p28 is Ca$^{2+}$-dependent. Recombinant Ral-A, generously supplied by Drs. Hiroshi Koide and Yoshito Kaziro and expressed in *Escherichia coli* by using the plasmid pGEX-2T, which contains the rat Ral-A cDNA, was also found to bind biotinylated calmodulin in the presence of 1 mM CaCl$_2$ (Fig. 4, lane 5). Recombinant Ral-A appears as a 25-kDa protein on 12% SDS-PAGE (Fig. 4, lane 6).

**Identification of a Putative Calmodulin-binding Domain in Ral-A—** A putative calmodulin-binding domain with the sequence SKEKNGKKKSKLSAKRIR arranged in an amphiphilic α-helix was identified in the C-terminal region of Ral-A at sequence position 183–200. Fig. 5 shows an axial helical wheel projection generated from the PLOT.A/HEL program based on the sequence of SKEKNGKKKSKLSAKRIR, corresponding to the putative calmodulin-binding domain of Ral-A. In this sequence there are four hydrophobic residues (Leu, Gly, Ile, and Ala) on one side of the wheel and nine strongly basic residues (Lys, Lys, Arg, and Lys, Lys, Lys, Lys, Arg, and Lys) segregated on the opposite side of the wheel. Although the contribution of the hydrophobic side of the amphiphilic helix may not be very high because of the interspersed charges, the overall features of two sets of adjacent positively charged residues, together with the hydrophobic residues on the other side, display the characteristic features of calmodulin-binding domains: a hydrophobic/basic composition with the propensity to form an amphiphilic helix.

The mean hydrophobic moment ($\langle \mu_H \rangle$) and the mean hydrophobicity ($\langle H \rangle$) of calmodulin-binding domains from various target enzymes and proteins were calculated according to Erickson-Viitanen and DaGrado (31). Fig. 6 shows the plot of the magnitude of the mean hydrophobic moment, $\langle \mu_H \rangle$, versus mean hydrophobicity, $\langle H \rangle$, which gives simultaneous visualization of the hydrophobicity and amphipathicity of a helix. Partitions define regions of calmodulin-binding domains: a hydrophobic/basic composition with the propensity to form an amphiphilic helix.

[**Fig. 2. Comparison of amino acid composition of p28 with Ral-A.** PVDF membrane-bound p28 was hydrolyzed in 6 M HCl. The individual amino acids were separated by reversed phase HPLC as described under “Experimental Procedures.” The relative abundance of each amino acid was determined by measuring its concentration compared with an internal standard. Amino acid composition of Ral-A is obtained from SWISS-PROT (accession number P11233). Open bars represent p28. Shaded bars represent Ral-A.]

[**Fig. 3. Guanine nucleotide binding of p28.** p28 was purified as described under “Experimental Procedures.” The GTP-binding activity of p28 was examined by photoaffinity labeling with $[\alpha^32P]GTP$ (A) and blot overlay with $[\alpha^32P]GTP$ after transferring to PVDF membrane (B). Panel A shows the autoradiogram of p28 photoaffinity labeled with $[\alpha^32P]GTP$ as detailed under “Experimental Procedures.” Lanes 1, 2, and 3 show various irradiation times: 0, 10, and 30 min. Lane 4 shows p28 photoaffinity labeled with $[\alpha^32P]GTP$ in the presence of 0.1 mM GTP at 30 min irradiation time. Panel B shows the autoradiogram of a p28 blot overlay with $[\alpha^32P]GTP$. Lanes 1 and 2 are molecular mass standards (M.W.) and p28 on the blot stained with Amido Black; lane 3 is the autoradiography of p28 labeled with $[\alpha^32P]GTP$; lanes 4 and 5 show the autoradiograms of p28 labeled with $[\alpha^32P]GTP$ in the presence of 0.1 mM GTP and 0.1 mM ATP. The position of p28 is indicated by an arrow.]
Identification of a calmodulin-binding domain in Ral-A.

A putative calmodulin-binding domain with the sequence SKEKNGKKKRKSLAKRIR was identified in the C-terminal region of Ral-A at sequence position 183–200. An axonal helical projection is generated from PLOT.A/HEL based on this sequence. Hydrophobic residues are boxed, and basic residues are depicted with a positive charge symbol.

the correspondence between the distribution of radioactivity and the stained bands. A radioactive peak was observed corresponding to the calmodulin band (17 kDa) in the presence of 1 mM CaCl₂, whereas no distinct radioactive peak was found in the presence of 1 mM EDTA. The results suggest that renatured calmodulin on PVDF membrane still retains its functional phosphate.

Lane: 1 2 3 4 5 6 7

FIG. 4. Detection of calmodulin-binding proteins by biotinylated calmodulin overlay. About 1–2 µg of purified p28 and recombinant Ral-A were subjected to SDS-PAGE and transferred to a PVDF membrane. The PVDF membranes containing p28 or recombinant Ral-A were blocked with blocking buffer and then incubated with 80 ng/ml of biotinylated calmodulin and 1 mM CaCl₂. In the control, 1 mM CaCl₂ was replaced by 5 mM EDTA in the blocking buffer in the above steps. The membranes were then incubated with 1:1000 diluted avidin-alkaline phosphatase (Sigma) in blocking buffer. The color development was achieved by incubating the membranes in a reaction mixture containing nitro blue tetrazolium and 0.05% 5-bromo-4-chloro-3-indolyl phosphate.

Lanes 1 and 2 show blots containing p28 overlaid with biotinylated calmodulin in the presence of Ca²⁺ and EDTA, respectively. Lanes 3 and 4 show blots containing p28 and molecular mass standards (M.W.) stained with 0.1% (w/v) Amido Black. Lane 5 shows the blot containing recombinant Ral-A overlaid with biotinylated calmodulin in the presence of Ca²⁺. Lanes 6 and 7 show blots containing recombinant Ral-A and molecular mass standards stained with 0.1% (w/v) Amido Black. p28 and recombinant Ral-A are indicated by arrows.

CaCl₂, the supernatant was removed, and the calmodulin-agarose resin sample was washed and eluted with an elution buffer containing 5 mM EDTA. Both supernatant and eluent were subjected to SDS-PAGE. As shown in Fig. 8, where lanes 2 and 3 represent control samples, p28 is observed in the eluent eluted with EDTA, whereas there is no p28 detected in the supernatant. The results indicate that p28 binds to calmodulin-agarose in a Ca²⁺-dependent manner and can be eluted by chelation of Ca²⁺ with EDTA. In the same experiments done in the presence of P18 (lane 4 and 5) and calmodulin (lane 6 and 7), p28 is observed both in the eluent and in the supernatant. This suggests that the binding of p28 with Ca²⁺/calmodulin is reduced in the presence of P18, similarly to that found in the presence of Ca²⁺/calmodulin itself. Thus, P18 can compete with p28 for binding to calmodulin in the presence of Ca²⁺, indicating that p28 and P18 share at least in part the same binding sites for calmodulin. However, under the conditions of the experiment, neither P18 nor calmodulin can completely prevent p28 binding to the calmodulin-agarose resin.

CD Spectroscopy Study of Calmodulin and Calmodulin-P18 Complex—To further characterize the calmodulin-binding domain in Ral-A, the binding of P18 to calmodulin was studied by CD spectroscopy. As shown in Fig. 9, P18 by itself has no obvious secondary structure but exists as a random coil in aqueous solution. However, calmodulin shows a CD spectrum typical of helical proteins, with minimum ellipticity at 222 and 208 nm (35). Upon the formation of calmodulin-P18 complex, a further decrease in negative mean residue ellipticity at 222 and 208 nm is observed. This suggests that addition of P18 to calmodulin at 1:1 ratio reduces to a significant extent the a-helical content in the complex. The CD spectroscopy results provide direct evidence for an interaction between P18 and calmodulin and show that this interaction results in a less a-helical content in the calmodulin-P18 complex (Fig. 9).

DISCUSSION

In this paper we have shown that a protein (p28) purified from Triton X-100 extracts of human erythrocyte plasma membrane by calmodulin affinity chromatography is highly related, if not identical, to Ral-A, a Ras-related GTP-binding protein, on the basis of interna peptide sequencing and amino acid composition analysis. This assignment was also confirmed by the characteristic of p28 to bind to [32P]GTP specifically and from
p28 was incubated with calmodulin-agarose resin in the presence of overnight. The samples were then centrifuged at 10,000 g and 6°C after p28 was incubated with calmodulin-agarose resin in the presence of P18. The supernatant and eluent, respectively, were prepared for SDS-PAGE analysis. The calmodulin-bound p28 was visualized by staining with 0.1% (w/v) Amido Black, as shown in the bottom panel. Blots to be counted for radioactivity were sliced transversely into 2-mm strips and then counted in a liquid scintillation analyzer.

Fig. 8. Binding of p28 to a calmodulin-agarose resin in the absence and in the presence of P18. Constant amounts of p28 and calmodulin-agarose resin were used in all experimental procedures. Samples of p28 (about 1.5 μg) were incubated with 0.5 ml of calmodulin-agarose resin in the buffer containing 1 mM CaCl₂ in the presence and in the absence of 10 μg of P18 or 10 μg of calmodulin (CaM) at 4°C overnight. The samples were then centrifuged at 10,000 g in a microcentrifuge, supernatants were removed, and proteins (referred to as supernatant) were prepared for SDS-PAGE analysis. The calmodulin-agarose resins were washed five times and then incubated with 1 ml of elution buffer containing 5 mM EDTA at 4°C for 5 h. After centrifugation at 10,000 g in a microcentrifuge, supernatants were removed, and proteins (referred to as eluent) were subjected to SDS-PAGE analysis. Lanes 1 and 2 show molecular mass standards (M.W.). Lanes 2 and 3 show samples obtained from the supernatant and eluent, respectively, after p28 was incubated with calmodulin-agarose resin. Lanes 4 and 5 show the supernatant and eluent, respectively, obtained after p28 was incubated with calmodulin-agarose resin in the presence of P18. Lanes 6 and 7 show the supernatant and eluent, respectively, obtained after p28 was incubated with calmodulin-agarose resin in the presence of calmodulin.

Fig. 7. Calmodulin blot overlay with [14C]P18. An 18-amino acid peptide (P18) with the sequence SKEKNGKKKKSKLAIR corresponding to a putative calmodulin-binding domain in Ral-A (p28) was synthesized and radioactively labeled with [14C]formaldehyde by the method of Rice and Means (33). Calmodulin (5 μg) was subjected to 6–16% SDS-PAGE and transferred to a PVDF membrane. One such blot containing calmodulin was incubated with 100 μl of [14C]P18 and 1 mM CaCl₂ in the washing buffer (50 mM Tris-HCl, pH 7.4, 0.5% (w/v) Tween 20) and the other was incubated with 100 μl of [14C]P18 and 1 mM EDTA in the washing buffer. After incubation for 30 min at room temperature, the blots were soaked in 20 ml of washing buffer and washed five times. The PVDF membrane-bound calmodulin was visualized by staining with 0.1% (w/v) Amido Black, as shown in the bottom panel. Blots to be counted for radioactivity were sliced transversely into 2-mm strips and then counted in a liquid scintillation analyzer.

Fig. 9. CD spectra of P18, calmodulin, and calmodulin/P18 mixture at 1:1 ratio. CD spectra were measured in 5 mM sodium borate buffer, pH 8.0, and 0.5 mM CaCl₂ with 6 μM calmodulin, or 6 μM P18, or calmodulin/P18 mixture at 1:1 ratio, respectively, as described under “Experimental Procedures.” For quantitative analysis, spectra of observed ellipticity, ε (mdeg) versus wavelength were expressed as spectra of calculated mean residue ellipticity, [θ]₂₅₀ (deg cm² mol⁻¹) versus wavelength. □ shows the CD spectra of 6 μM calmodulin (CaM); ○ shows the CD spectra of calmodulin/P18 mixture at 1:1 ratio; ● shows the CD spectra of 6 μM P18.

A Calmodulin-binding Domain in Ral-A

Although the Ral-A protein purified from human erythrocyte membrane has an apparent molecular mass of approximately 28,000 Da on SDS-PAGE, the full length human Ral-A cDNA encodes a protein with a predicted molecular mass of 23,567 Da. The discrepancy of molecular mass may be due to post-translational modification. In agreement with our finding, the Ral-A gene products isolated from human platelet membrane have molecular mass of 28 kDa (36), whereas the human Ral-A gene protein expressed in E. coli is a 29-kDa protein (37). We have interpreted this as evidence that Ral-A binds to plasma membrane through groups associated with its post-translational modification. Ral proteins undergo sequential post-translational modifications that occur at a CAAL motif in the C-terminal region of the molecules (38). Ral-A translation products in reticulocyte lysates have been found to be modified by 20-carbon isoprenyl groups (39). Very recently, post-translational modification of Ral has been shown to enhance the activities of RalGDS, indicating a functional role of this modification for transmitting its signal effectively (40).

A striking and novel finding in our study is that Ral-A has been identified as a calmodulin-binding protein. The interaction with calmodulin was first detected on the basis of the ability of Ral-A to bind to a calmodulin affinity column in a Ca²⁺-dependent manner and to be released upon elution by chelation of Ca²⁺ with EDTA. The indication of Ral-A binding to calmodulin was strongly supported by further evidence that calmodulin could block Ral-A phosphorylation by cAMP-dependent protein kinase, cGMP-dependent protein kinase, and Ca²⁺/phospholipid-dependent protein kinase. Moreover, biotinylated calmodulin overlay experiments have shown that biotinylated calmodulin binds to renatured Ral-A in a Ca²⁺-dependent manner, confirming our finding that Ral-A is a calmodulin-binding protein. After these results were completed, it was reported that two other Ras-related GTP-binding protein groups, Kir/Gem and Rin, contain calmodulin-binding domains in their extended polybasic C-terminal regions (23, 24). However, unlike Ral-A, Kir/Gem and Rin lack a typical CAAX isoprenylation motif in their C termini.

Based on inspection of Ral-A sequence with respect to the presence of a basic/hydrophobic composition with the propen-
sity to form an amphiphilic helix, a putative calmodulin-binding domain (P18) with the sequence SKEKNGKKRKSLAKRIR was identified within the C-terminal region of Ral-A. The α-helical structure of this domain shows the typical basic, amphiphilic features that are consistent with the criterion for the calmodulin-binding peptides established by DeGrado and co-workers (31, 41). This amphiphilic helix model has now been demonstrated to underlie the calmodulin-binding properties common to a variety of peptides by NMR and CD spectroscopy studies (42–45). The most unusual feature of the calmodulin-binding domain identified in Ral-A is that it is rather more hydrophilic than hydrophobic, because it contains 9 of 18 hydrophilic amino acids (50%) but only four hydrophobic residues (22%). By contrast, the mastoparan, high affinity calmodulin-binding peptides, have 21–28% hydrophilic amino acids and 36–50% hydrophobic residues (46). Although it has been established that the interaction of calmodulin with its target proteins is predominantly hydrophobic, it is complemented by acidic side chains from the calmodulin EF-hand interacting with basic residues of the target proteins (47). For instance, the high affinity calmodulin-binding site (Kd = 20 nM) of the Na+/H+ exchange isoform 1 with the segments of RNNLQK-TRQRIRSYNRHT contains 7 of 18 hydrophilic amino acids (39%) but only 2 hydrophobic residues (11%) (48). The replacement of 4 positively charged residues with negative or neutral ones is found to inhibit the binding of this region to calmodulin. The characteristic of the calmodulin-binding domain in Ral-A is reminiscent of domains such as those in Na+/H+ exchange isoform 1. This indicates that apart from the hydrophobic interaction, the binding of calmodulin with target proteins involves strong electrostatic interaction, which arises from the charged residues of the target peptide interacting with acidic side chains from calmodulin EF-hand motifs.

The calmodulin-binding domain in Ral-A is somewhat hydrophilic, with the mean hydrophobicity (Hb) = –1.08 kcal/mol and hydrophobic moment (μHb) = 0.31, suggesting that it belongs to the globular helices group. According to Erickson-Vitanen and DeGrado (31), several calmodulin-binding peptides with dissociation constants of less than 5 nM have been reported to fall in the most amphiphilic “surface seeking” region, with a range of (Hb) values of 0 to 0.4 kcal/mol and values of (μHb) of 0.5 or greater. However, this criterion is only suitable for those calmodulin-binding peptides with the sequence of 12 residues and an overall net positive charge above +4. Considering the lack of amino acid sequence homology and the substantial difference in length in calmodulin-binding peptides, (μHb) and (Hb) for various calmodulin-binding peptides with available sequences were calculated. According to our calculation, the hydrophobic moment plot shows that P18, as well as most calmodulin-binding domains, tend to fall into the globular helices region with the range of (Hb) from –1.0 to 0.5 kcal/mol and the range of (μHb) from 0.1 to 0.7. Despite its usefulness of visualizing the hydrophobicity and amphiphilicity of a calmodulin-binding domain, the hydrophobic moment plot cannot be used for prediction of calmodulin-binding sequences because it ignores the contribution of basicity to calmodulin binding, and there is no predictable relation of the calculated hydrophobic moment to the affinity constants of different calmodulin-binding peptides (31).

Further characterization of this proposed calmodulin-binding domain in Ral-A was undertaken by using a synthetic peptide (P18) corresponding to the sequence H-SKEKNGKKRKSLAKRIR-OH and three independent methods: calmodulin overlay with 14C-labeled P18, a Ral-A competitive binding study with calmodulin in the absence and in the presence of P18, and CD spectroscopy study of calmodulin-P18 complex. We have shown that P18 interacts with calmodulin in a Ca2+-dependent manner, thereby further supporting its role as a calmodulin-binding domain. It has been reported for numerous calmodulin-binding peptides that a significant increase in negative mean residue ellipticity at 208 and 222 nm is observed, indicating the formation of additional α-helical structure upon calmodulin-peptide complex formation (31, 44, 45, 49). Because in the presence of a target peptide the long central helix, which connects two globular domains in calmodulin, is disrupted and unravelled to accommodate peptide binding, Ca2+/calmodulin generally does not gain secondary structure (42, 43, 50). Thus, the increased α-helical content is exclusively attributed to binding of the peptide to calmodulin. In contrast, CD studies of the calmodulin-P18 complex show a decrease in negative mean residue ellipticity at 208 and 222 nm, indicating that the interaction of P18 with Ca2+/calmodulin results in less helical content in calmodulin-P18 complex. This behavior closely resembles that of Phk13, one of the high affinity (Kd = 5 nM) calmodulin-binding domains in the γ subunit of glycogen phosphorylase kinase (52). The addition of Phk13 to calmodulin also results in a decrease in negative mean residue ellipticity at 222 and 208 nm. The structure of calmodulin-Phk13 complex has also been studied by tryptic digestion and radiationless energy transfer measurements and a model of calmodulin interacting with Phk13 has been proposed in which calmodulin has an extended conformation resembling its crystallographic structure, whereas Phk13 exists in a nonhelical conformation, making contact with both the N- and C-terminal lobes and lying roughly parallel to the long axis of calmodulin (52). Whether the interaction of P18 with calmodulin will match this model needs further structure studies. It is very difficult to give a precise interpretation based on CD data alone because the diagnostic α-helical bands for the protein and the peptide overlap.

Calmodulin is a highly conserved, small (148 amino acid), acidic Ca2+-binding protein. It is considered to be the major regulator of Ca2+-dependent signaling pathways in eukaryotic cells, mediating a wide variety of physiological processes, including glycogen metabolism, secretion, muscle contraction, and cell division (53, 54). The regulatory effects induced by calmodulin are mediated by numerous calmodulin-binding proteins. Calmodulin-binding proteins therefore constitute a major group of signal transducing proteins. The studies presented here display new experimental evidence that Ral-A is calmodulin-binding protein. This finding has provided the new possibility of modulation of Ral-A by Ca2+ or a functional role of Ral-A protein in Ca2+ signaling pathways. Although it has been firmly established that heterotrimeric G proteins are involved in Ca2+ release through the regulation of phospholipase C, adenylate cyclase and K+ channels (55), there is an increasing amount of information on the involvement of low molecular mass GTP-binding proteins in intracellular Ca2+ mobilization. Rap1 has been found to regulate platelet plasma membrane Ca2+ transport (56). As for Ca2+ release from the intracellular store, Chong et al. (57) have shown that activation of Rho pathways stimulate production of a phospholipase C substrate, thereby enabling Ca2+ release triggered by platelet-derived growth factor. In addition, Rho has been shown to play a role in helping to decrease increased Ca2+ levels without affecting the machinery for Ca2+ release from inositol 1,4,5-trisphosphate-dependent Ca2+ stores (58). A Rac-dependent Ca2+ influx pathway has also been reported (51). This accumulating evidence, together with our finding that Ral-A interacts with Ca2+/calmodulin, shows that Ras-related GTP-binding proteins may be modulated by Ca2+ and may have a potentially significant function in Ca2+ signaling pathways. It also raises the possi-
bility of cross-talk between signal transduction pathways mediated by Ca^{2+}/calmodulin and Ras proteins.

Acknowledgments—We thank Dr. Ken Mitchelhill and Dr. Bruce F. Kemp (St. Vincent’s Institute of Medical Research, Melbourne, VIC, Australia) for peptide sequencing and protein identification. We also thank Jun X. Yan (Macquarie University Center for Analytical Biotechnology, Sydney, NSW, Australia) for protein amino acid composition analysis. We are grateful to Dr. Jose Martin-Nieto and Dr. Antonio Villalobo (Instituto de Investigaciones Biomedicas, Madrid, Spain) and J. M. Toy Weiss (Department of Biochemistry, University of Sydney) for performing computer based searches for putative calmodulin-binding domains. We thank Drs. Hiroshi Koide and Yoshito Kaziro (Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, Tokyo, Japan) for supplying us recombinant Ral-A. We thank Drs. Hiroshi Koide and Yoshito Kaziro (Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, Tokyo, Japan) for supplying us recombinant Ral-A. We thank Dr. Ken Mitchelhill and Dr. Bruce F. Kemp (St. Vincent’s Institute of Medical Research, Melbourne, VIC, Australia) for peptide sequencing and protein identification. We also thank Jun X. Yan (Macquarie University Center for Analytical Biotechnology, Sydney, NSW, Australia) for the peptide sequencing and protein identification. We thank Delia Leung for establishing purification methods for Ral-A and modulin-binding properties of p28 and its phosphorylation properties. We thank Dr. Ken Mitchelhill and Dr. Bruce F. Kemp (St. Vincent’s Institute of Medical Research, Melbourne, VIC, Australia) for peptide sequencing and protein identification. We also thank Jun X. Yan (Macquarie University Center for Analytical Biotechnology, Sydney, NSW, Australia) for the peptide sequencing and protein identification. We thank Delia Leung for establishing purification methods for Ral-A and modulin-binding properties of p28 and its phosphorylation properties. We thank Delia Leung for establishing purification methods for Ral-A by DEAE-cellulose column chromatography.

REFERENCES
1. Kaziro, Y., Itsh, H., Kozasa, T., Nakafuku, M., and Satoh, T. (1991) Annu. Rev. Biochem. 60, 349–400
2. Bokoch, G. M., and Der, C. J. (1993) FASEB J. 7, 750–759
3. Marshall, M. S. (1995) FASEB J. 9, 1311–1318
4. Cardin, P., and Tavitian, A. (1986) EMBO J. 5, 2201–2208
5. Cardin, P., and Tavitian, A. (1989) Nucleic Acid Res. 17, 4380–4385
6. Polakis, P. G., Weber, R. F., Nervio, B., Didsbury, J. R., Evans, T., and Snyderman, R. (1989) J. Biol. Chem. 264, 16385–16389
7. Olofsson, B., Chardin, P., Touloukh, N., Zahrnau, A., and Tavitian, A. (1988) Oncogene 3, 231–234
8. Wider, G. M., Vagelosrapu, M., Rim, S., and Denker, J. K. (1993) J. Biol. Chem. 268, 451–559
9. Bielinske, D. F., Pyun, H. Y., Linko-Stentz, K., Macara, I. G., and Fine, R. E. (1992) J. Biol. Chem. 267, 181–188
10. Volkan, W., Pfeffer, J., Eriken, L. A., and Scheller, R. H. (1993) FEBS Lett. 317, 53–56
11. Emery, R., Freedman, S., and Feig, L. A. (1991) J. Biol. Chem. 266, 9703–9706
12. Bhullar, R. P., Chardin, P., and Haslam, R. J. (1990) FEBS Lett. 260, 48–52
13. Farrnsworth, C. L., and Feig, L. A. (1991) Mol. Cell. Biol. 11, 4822–4829
14. Kinsella, B. T., Erdman, R. A., and Maltese, W. A. (1991) J. Biol. Chem. 266, 9786–9794
15. Reese, J. H., and Maltese, W. A. (1991) Mol. Cell. Biochem. 104, 109–116
16. Hinoi, T., Kishida, S., Koyama, S., Ikeda, M., Matsuura, Y., and Kikuchi, A. (1996) J. Biol. Chem. 271, 19710–19716
17. O’Leary, K. T., and DeGrado, W. F. (1990) Trends Biochem. Sci. 15, 59–64
18. Ikura, M., Clore, G. M., Gronenborn, A. M., Zhu, G., Klee, C. B., and Bax, A. (1992) Science 256, 632–638
19. Meador, W. E., Means, A. R., and Quiocho, F. A. (1993) Science 262, 1718–1721
20. Zhang, M., and Vogel, H. J. (1994) Biochemistry 33, 1614–1717
21. Yuan, T., Mietzner, T. A., Montelaro, R. C., and Vogel, H. J. (1995) Biochemistry 34, 109042
22. McDowell, L., Sanyal, G., and Prendergast, F. G. (1985) Biochemistry 24, 2799–2804
23. Ikura, M. (1996) Trends Biochem. Sci. 21, 14–17
24. Bertrand, B., Wakabayashi, S., Ikeda, T., Poussysegure, J., and Shigekawa, M. (1994) J. Biol. Chem. 269, 15703–15709
25. Cox, J. A., Comte, M., Fitton, J. E., and DeGrado, W. F. (1985) J. Biol. Chem. 260, 2527–2534
26. Roth, S. M., Schneider, D. M., Strobel, L. A., Van Berkum, M. F., Mians, A. R., and Wand, A. J. (1991) Biochemistry 30, 10078–10084
27. Peppelenbosch, M. P., Terpelen, L. G. J., Devries-Smits, A. M. M., Gru, R. G., Mfabe, L., Symons, M. H., DeLaat, S. W., and Bos, J. J. (1996) J. Biol. Chem. 271, 7863–7868
28. Juminaga, D., Albaugh, S. A., and Steinier, R. F. (1994) J. Biol. Chem. 269, 1660–1667
29. Vogel, H. J. (1994) Biochem. Cell Biol. 72, 357–376
30. Vogel, H. J., and Zhang, M. (1995) Mol. Cell. Biochem. 149/150, 3–15
31. McPherson, D. R. (1995) Cell 80, 259–268
32. Corvera, E., Zoef, J. P., Gunzburg, J. D., Tavitian, A., and Levy-Tolmado, S. (1992) Biochem. J. 281, 325–331
33. Chong, L. D., Traynor-Kaplan, A., Bokoch, G. M., and Schwartz, M. A. (1994) Cell 79, 507–513
34. Katz, N. (1996) Biochem. Biophys. Res. Commun. 226, 580–584