A Novel Endoprotease Responsible for the Specific Cleavage of Transducin γ Subunit†

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ABSTRACT: Isoprenylated/methylated heterotrimeric G proteins play important roles in a large number of signal transduction processes. While the enzymology of isoprenylated/methylated protein biosynthesis is well understood, nothing is known about how these proteins are degraded. In this article, a novel endoproteolytic activity has been identified from bovine retina and is shown specifically to remove the glycylfarnesylcysteine moiety from the carboxyl terminus of T_γ. When tested in a GTP binding assay, freshly prepared proteolyzed $T_{\beta\gamma}$ was unable to catalyze the binding of guanosine 5′-(γ-thio)triphosphate (GTP-γ-S) to T_α in the presence of detergent solubilized rhodopsin. The optimum pH for this proteolytic activity is approximately 6, and the pH profile corresponds to an enzyme having pK_a's of 4.4 ± 0.1 and 7.7 ± 0.1 for its active site residues. After analyzing a series of protease inhibitors, we found E-64, a specific thiol protease inhibitor, to be the most effective irreversible inhibitor of this enzyme, suggesting that the endoprotease might be a thiol protease. Affinity labeling studies using biotinylated affinity labeling probes have identified a 35 kDa protein as a candidate for the endoprotease.

Heterotrimeric G proteins are isoprenylated and methylated on the carboxyl terminal cysteine residue of their γ subunits (Lai et al., 1990; Fukada et al., 1990; Yamane et al., 1990). These hydrophobic posttranslational modifications appear to be essential for the full manifestation of the activity of the proteins so modified (Inglese et al., 1992; Hancock et al., 1989; Ohguro et al., 1991). The biological function of these modifications appears to largely lie in increasing the hydrophobicity of the modified proteins, enhancing their abilities to become membrane associated (Hancock et al., 1991; Schafer & Rine, 1992; Muntz et al., 1992; Silvius & l'Heureux, 1994). Possible specific effector roles for the isoprenylated/methylated cysteine moieties of γ subunits have also been discussed (Clapham & Neer, 1993; Sternweis, 1994; Iñiguez-Lluhi et al., 1992; Koch et al., 1993; Marshall, 1993; Parish et al., 1995).

The enzymes required for the biosynthesis of isoprenylated/methylated heterotrimeric G proteins have been well studied. The γ subunits, which terminate in a CAAX motif (C = cysteine, A = aliphatic amino acid and X = any amino acid), are isoprenylated at C (Yamane et al., 1990; Mumby et al., 1990; Lai et al., 1990; Sinensky & Lutz, 1992), cleaved by a prenyl-specific endoprotease to remove AAX (Ma & Rando, 1992; Ashby et al., 1992), and finally carboxymethylated at the newly exposed carboxylate of cysteine (Pérez-Sala et al., 1991; Clarke, 1992; Gilbert et al., 1992). By comparison, virtually nothing is known about the catabolism of isoprenylated/methylated proteins. It is interesting to note that isoprenylated cysteine derivatives are biologically quite active (Scheer & Gierschik, 1993; Philips et al., 1993; Ding et al., 1994; Ma et al., 1994), suggesting that a specific mechanism needs to be in place to process these derivatives, should they be generated in cells. Further, prenylated/methylated cysteine molecules appear to be ligands of the multidrug resistance transporter, an integral membrane protein responsible for the removal of anticancer drugs from drug-resistant tumor cells (Zhang et al., 1994).

In this article, a proteolytic activity is described from retinal rod outer segments (ROS)[1] capable of proteolytically cleaving the γ subunit of the heterotrimeric G protein, retinal transducin (T). T_γ, which is farnesylated/methylated at its carboxyl terminal cysteine residue, terminates in the sequence GGC (Pérez-Sala et al., 1991; Fukada et al., 1990). The endoprotease cleaves the protein between the two glycines, producing glycylfarnesylcysteine methyl ester (GFCM) and the remainder of the T_γ subunit. This endoproteolytic activity is probably the one responsible for the degraded, inactive $T_{\beta\gamma}$ found during the isolation of transducin (Ohguro et al., 1991; Parish & Rando, 1994; Bigay et al., 1994) and for the observed instability of partially purified transducin. Freshly prepared endoproteolyzed $T_{\beta\gamma}$ is inert biochemically when the ability of T_α to bind GTP-γ-S is assayed in the presence of $T_{\beta\gamma}$ and rhodopsin in detergent, confirming the importance of isoprenylation for the activity of transducin (Ohguro et al., 1991).

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[1] Abbreviations: AdoMet, *S*-adenosylmethionine; avidin-HRP, horseradish peroxidase conjugated avidin; BBB-E-64, *N*-(*N*$^\alpha$-4-benzoylbenzoyl-L-biocytinoyl)-*N*′-(E-64)-1,6-diaminohexane chloromethyl ketone; BBB-FPR CMK, *N*-(*N*$^\alpha$-4-benzoylbenzoyl-L-biocytinoyl)-*N*′-Phe-Pro-Arg chloromethyl ketone; E-64, *N*-(*trans*-epoxysuccinyl)-L-leucine 4-guanidinobutylamide; FCM, farnesylcysteine methyl ester; G protein, GTP binding protein; GFCM, glycylfarnesylcysteine methyl ester; GTP-γ-S, guanosine 5′-(γ-thio)triphosphate; HCl-FPR CMK, HCl-Phe-Pro-Arg chloromethyl ketone; PNB, *p*-nitrobenzyl; ROS, rod outer segment; streptavidin−HRP, horseradish peroxidase conjugated streptavidin; $T_{\beta\gamma}$-OCH_3, transducin $\beta\gamma$ subunit methylated at the C-terminus of the γ subunit; $T_{\beta\gamma}$-OH, unmethylated transducin $\beta\gamma$ subunit; TFA, trifluoroacetic acid; ECL, enhanced chemiluminescence.

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MATERIALS AND METHODS

Materials

Frozen bovine retinas were obtained from J. A. & W. L. Lawson Co. (Lincoln, NE). Avidin-conjugated horseradish peroxidase (avidin−HRP), biotinylated molecular weight markers, and Bio-Rad ^{D}C protein assay reagents were from Bio-Rad. DAIICHI silver stain-II was purchased from Integrated Separation Systems. Nitrocellulose (45 μm pore size) was from Schleicher and Schuell. The luminol ECL Western-blotting detection reagents were from Amersham Life Sciences. Hydrofluor scintillation fluid was from National Diagnostics. Hexylagarose was from Sigma. Blue sepharose CL-6B was from Pharmacia.

GTP, GTP-γ-S, and protease inhibitors including E-64, leupeptin, trypsin inhibitor, *N*-tosyl-L-lysine chloromethyl ketone (TLCK), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), pepstatin, and 4-(amidinophenyl)methane-sulfonyl fluoride (APMSF) were from Boehringer Mannheim. HCl-FPR CMK was from Sigma. *N*,*N*-Diisopropylethylamine (Hünig's base) was from Aldrich. GTP-γ-^{35}S (1340 Ci mmol^{-1}) and *S*-[methyl-^{3}H]-adenosyl-L-methionine ([^{3}H]-AdoMet, 40 Ci mmol^{-1}) were from NEN/Dupont. N^{α}-(4-Benzoylbenzoyl)-L-biocytin and 1-(*N*-(N^{α}-(4-benzoylbenzoyl)-L-biocytinoylamino)-6-(*N'*-boc-amino)hexane was prepared according to previously published procedure (Gilbert & Rando, 1995). Farnesylcysteine methyl ester was prepared according to a published procedure (Ma et al., 1994).

Methods

Isolation of Transducin and Proteolytic Activity. Transducin subunits, T_{α} and $T_{\beta\gamma}$, were prepared from bovine retinas (50) as described previously (Parish & Rando, 1994). In brief, a crude transducin preparation was obtained by washing the isolated ROS membranes twice (total 75 mL) with buffer A (10 mM Tris·HCl, pH 7.4, containing 1 mM DTT and 0.1 mM EDTA) containing 50 μM GTP. The proteolytic activity was separated from this sample by hexylagarose chromatography (250 × 10 mm), further purifying the transducin heterotrimer (Fung et al., 1981). The flow rate of the column was 25 mL·h^{-1}, and 3-mL fractions were collected. A gradient of 20−200 mM NaCl in buffer B (10 mM MOPS, pH 7.4, containing 1 mM DTT and 1 mM EDTA; total volume of gradient = 150 mL) resulted in the separation of the proteolytic activity in a broad band as monitored by UV absorbance at 280 nm. This peak eluted from the column between 20 and 40 mM NaCl. The transducin peak, which eluted from the hexylagarose column between 50 and 80 mM NaCl, was identified by monitoring the ability of these fractions to bind GTP-γ-S in the presence of photoactivated rhodopsin. The fractions containing the proteolytic activity were incapable of binding GTP-γ-S. The fractions that contained protein by UV absorbance but did not bind GTP-γ-S were combined and concentrated to a small volume with a micro-ultrafiltration system using a PM-10 membrane (Amicon). The concentrated solution was then used as a source of protease. The transducin fractions were concentrated and separated into T_{α} and $T_{\beta\gamma}$ subunits by chromatography with blue sepharose CL−6B, as described previously (Parish & Rando, 1994), (final buffer for T_{α} and $T_{\beta\gamma}$: buffer A containing 100 mM NaCl and 5 mM $MgCl_2$).

Analysis of GTP Binding Activity. The binding of GTP-γ-S to T_{α} was performed as described previously (Parish & Rando, 1994). Rhodopsin was solubilized in dodecylmaltoside (Longstaff & Rando, 1985). $T_{\beta\gamma}$ was proteolyzed for 16 h as described below. To ensure that no residual $T_{\beta\gamma}$-OCH_3 remained in this sample, the NaCl concentration was reduced to less than 1 mM by sequential dilution with buffer A and filtration with a Microcon 10 concentrator (Amicon). At this NaCl concentration, $T_{\beta\gamma}$-OCH_3 binds to blue sepharose while cleaved $T_{\beta\gamma}$ does not adhere. The cleaved $T_{\beta\gamma}$ was removed from the blue sepharose after centrifugation (5 min, 16 000*g*). The resin was washed with buffer A containing 5 mM $MgCl_2$, and the combined supernatants were concentrated with a Centricon 10 concentrator (Amicon). A control sample was treated in an analogous manner except that it was treated with neither the proteolytic activity nor the blue sepharose. A second sample was treated with the proteolytic activity but not with blue sepharose.

Preparation of Radiolabeled $T_{\beta\gamma}$-[^{3}H]-OCH_3. Nonradioactive $T_{\alpha\beta\gamma}$ (0.76 mg) in 200 mM Na-HEPES, pH 7.40, containing 100 mM NaCl and 5 mM $MgCl_2$ (40 μL) was incubated with 1 μL extensively washed ROS membrane (27.3 mg mL^{-1}) containing methyltransferase activity (Gilbert et al., 1992) and [^{3}H]-AdoMet (4.9 mCi, 40.4 Ci mmol^{-1}) in a 37 °C shaker. After 3 h incubation, the sample was centrifuged at 16 000*g* (4 °C, 6 min), and the obtained supernatant contained some $T_{\alpha\beta\gamma}$-[^{3}H]-OCH_3. Membrane-bound transducin was extracted with 2 × 60 μL GTP (50 μM). All supernatants containing $T_{\alpha\beta\gamma}$-[^{3}H]-OCH_3 were then combined and treated with blue sepharose CL-6B (20 μL) to separate T_{α} from $T_{\beta\gamma}$-OCH_3. The gel was preequilibrated with buffer A containing 100 mM NaCl and 5 mM $MgCl_2$. In the presence of 100 mM NaCl, T_{α} binds to blue sepharose while $T_{\beta\gamma}$-OCH_3 does not. The eluted $T_{\beta\gamma}$-OCH_3 was concentrated, resulting in the $T_{\beta\gamma}$-[^{3}H]-OCH_3 solution (400 μL, 0.95 mg mL^{-1}, 2.4 mCi mmol^{-1}), which is used in the following proteolytic activity assays.

Ac-LKGGC(farnesyl)-[^{3}H]-OCH_3 was prepared from Ac-LKGGC(farnesyl)-OH according to a previously published procedure (Pérez-Sala et al., 1991). Ac-LKGGC(farnesyl)-OH was prepared as described below.

HPLC Analysis I. This analysis was based on a previously published procedure (Parish & Rando, 1994). The proteolysis of transducin γ subunit was monitored at 205 nm by reverse-phase HPLC analysis using a C18 column (Dynamax 300 Å, Rainin). (For all HPLC, reservoir A = 10 mM TFA in water, reservoir B = 10 mM TFA in acetonitrile.) After 10 min at 5% B, a linear gradient (0.75 mL min^{-1}) was run from 5% to 95% B over 40 min. Peaks corresponding to T_{γ}-OCH_3 and the cleaved transducin fragment T_{γ} eluted at 41 and 36 min, respectively. Retention times varied within 1−2 min depending on column and HPLC conditions. T_{β} does not elute from the column under the three HPLC conditions described in this paper. All samples were injected with 3 M guanidinium chloride. Experiments involving the heat sensitivity of the protease, the time course of cleavage, and the proteolysis of unmethylated transducin ($T_{\beta\gamma}$-OH), were carried out using this analysis.

HPLC Analysis II. This analysis was designed for the simultaneous identification of radiolabeled transducin (T_{γ}-[^{3}H]-OCH_3) and cleaved GFC-[^{3}H]-M. The analysis was accomplished by reverse-phase HPLC using a C18 column (Dynamax 300 Å, Rainin). UV absorbance was monitored at 205 nm. A linear gradient (0.75 mL min^{-1}) was run from 45% to 95% B over 20 min. Each fraction (0.75 mL) was

collected and counted in scintillation fluid (10 mL) using a Beckman LS-1800 scintillation counter. This gradient completely separated T_γ-[^{3}H]-OCH_3 (12.5 min) and the cleaved radioactive fragment GFC-[^{3}H]-M (15.6 min).

HPLC Analysis III. This analysis was designed to separate FCM and GFCM, the two possible proteolytic products. The analysis was accomplished by reverse-phase HPLC using a C8 column (Dynamax 300 Å, Rainin). A nonlinear gradient (curve 2, Waters 600E System Controller) at 0.2 mL min^{-1} was run from 45% to 60% B over 40 min followed by constant 60% B (20 min). Radiolabeled transducin $\beta\gamma$ ($T_{\beta\gamma}$-[^{3}H]-OCH_3) was used as the substrate. The identity of the proteolytic product was determined by the elution of the radiolabeled product with unlabeled FCM and GFCM, which had been coinjected.

SDS–PAGE and Western Blotting Analysis. The conditions used for SDS–PAGE and Western-blotting analysis of BBB-FPR CMK and BBB-E-64 labeled endoprotease were almost identical to those reported for the detection of aspartic proteases (Gilbert & Rando, 1995), except that streptavidin–HRP was applied instead of avidin–HRP for the experiment with BBB-E-64.

Miscellaneous Procedures. Protein concentrations were determined by Bio-Rad ^DC Protein Assay. Molecular mass of proteolyzed T_γ subunit was determined by electrospray ionization mass spectrometry at the Harvard Microchemistry Facility or by matrix assisted laser desorption mass spectrometry with a Millipore PM^3-1000 using α-cyano-3-hydroxycinnamic acid as the matrix.

Syntheses

Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy was obtained with a Varian VRX 500S Spectrometer operating at a proton frequency of 500 MHz. FABMS spectra were obtained on a Finnegan 4000 mass spectrometer. All amino acids are L-enantiomers.

Glycylfarnesylcysteine Methyl Ester (GFCM). Farnesylcysteine (371 mg, 1.1 mmol) was partially dissolved in 1:1:1 DMF:dioxane:10% Na_2CO_3 (15 mL) with stirring at 25 °C. Fmoc-glycine pentafluorophenyl ester (460 mg, 1.0 mmol) was added to the suspension and stirring was continued for 15 h, over which time all starting material dissolved. Ethyl acetate (50 mL) was added to the reaction mixture, and the organic layer was washed with 1 N HCl and brine, dried ($MgSO_4$), and concentrated to an oil. Silica gel chromatography (2:1 hexane:ethyl acetate, then 1:1 ethyl acetate:methanol) provided the desired Fmoc-glycylfarnesylcysteine (Fmoc-GFC) as a colorless oil (255 mg, 43%): ^{1}H NMR (DMSO-d_6) δ 7.86 (2H, d, J = 8.0 Hz, Fmoc), 7.69 (2H, d, J = 7.5 Hz, Fmoc), 7.66 (1 H, m, -NH-), 7.39 (2 H, t, J = 7.0 Hz, Fmoc), 7.31 (2 H, t, J = 7.0 Hz, Fmoc), 5.11 (1 H, t, J = 7.0 Hz, vinyl-H), 5.03 (2 H, m, vinyl–H), 4.23 (2 H, m, Fmoc-CH_2-), 4.21 (1H, m, Cys-C_α-H), 4.06 (1H, m, Fmoc-CH-), 3.61 (2H, pdd, J = 16.5, 5.5 Hz, Gly-C_α-H_2), 3.08 (2H, m, farnesyl-CH_2-S), 2.87 (1H, m, Cys-C_β-H), 2.70 (1H, m, Cys-C_β-H), 2.00–1.85 (8H, m, farnesyl CH_2), 1.60 (3H, s, vinyl methyl), 1.57 (3H, s, vinyl methyl), 1.52 (6H, s, vinyl methyls); FABMS 603 (glycerol, [M-H]$^-$).

Fmoc-GFC was deprotected by treatment with 10% piperidine in DMF for 2 h at 25 °C. The reaction was lyophilized to dryness, providing a solid that was soluble in 0.1 N HCl and was purified by HPLC on a C18 column (Dynamax 300 Å, Rainin). The desired product (GFC) eluted after 16 min when a linear gradient was run at 0.75 mL min^{-1} from 45% to 95% B over 20 min; FABMS 383 ([M+H]$^+$).

GFCM was prepared by treating the HPLC purified sample of GFC with trimethylchlorosilane in methanol. The crude product was then purified by HPLC on a C18 column using the same gradient described for GFC; FABMS 397 ([M+H]$^+$).

Farnesylated Penta- and Decapeptides. Peptides were prepared having the sequence NPFKELKGGC and LKGGC, corresponding to the carboxyl terminus of T_γ. These peptides were prepared using standard solid–phase peptide synthesis (Millagen/Biosearch 9600 Synthesizer) with *N*-α-Fmoc amino acids on a Wang resin. Before removal from the resin, each peptide was acetylated at the amino terminus with acetic anhydride. Farnesylation was performed according to the procedure of Xue et al. (1992). The desired products were isolated by HPLC on a C18 column. The decapeptide sequence contains an internal glutamate residue which complicates the synthesis of its carboxyl terminal methyl ester. For the preparation of the decapeptide methyl ester, the glutamic acid side chain carboxylate must be protected to prevent its methylation. In place of the standard Fmoc-Glu(OtBu)-OH, Fmoc-Glu(OPNB)-OH was utilized. The PNB group is not cleaved during TFA removal of the peptide from the resin. After amino terminal acetylation and TFA treatment, the decapeptide was farnesylated, and then methylated with HCl/methanol. Lastly, the PNB group was removed with Zn/AcOH. Some diester was observed, but HPLC purification provided a sample of the desired monomethyl ester (Dynamax 60Å semi-prep column, Rainin, 35–95% B over 20 min, decapeptide-OH: 15 min; decapeptide-OCH_3: 17 min); FABMS Ac-NPFKELKGGC-(farnesyl)-OH 1338 ([M+H]$^+$); Ac-NPFKELKGGC-(farnesyl)-OCH_3 1352 ([M+H]$^+$); Ac-LKGGC(farnesyl)-OH 723 ([M+H]$^+$); intermediates: Ac-NPFKE(OPNB)LKGGC-(farnesyl)-OH 1473 ([M+H]$^+$); Ac-NPFKELKGGC-OH 1134 ([M+H]$^+$).

Fmoc-Glu(OPNB)-OH. To a solution of Fmoc-Glu(OH)-OtBu (902 mg, 2.1 mmol) in DMF (20 mL) was added sodium bicarbonate (350 mg, 4.2 mmol), 4-nitrobenzyl chloride (1.08 g, 6.3 mmol), and potassium iodide (700 mg, 4.2 mmol). After stirring at 25 °C for 24 h, the reaction was poured onto water (50 mL) and ethyl acetate (30 mL). The organic layer was removed and the aqueous was extracted further with ethyl acetate (3 × 20 mL). The combined organic layers were dried ($MgSO_4$) and concentrated to an orange oil. The crude product was purified by silica gel chromatography (4:1 hexane:ethyl acetate, then 2:1 hexane:ethyl acetate), providing the desired Fmoc-Glu-(OPNB)-OtBu (660 mg, 56%): TLC (2:1 hexane:ethyl acetate containing 1% acetic acid) R_f Fmoc-Glu(OH)-OtBu 0.24, Fmoc-Glu(OPNB)-OtBu 0.50; ^{1}H NMR ($CDCl_3$) δ 8.18 (2H, d, J = 8.5 Hz, PNB aryl), 7.75 (2H, d, J = 8.0 Hz, Fmoc), 7.58 (2H, dd, J = 7.0, 6.0 Hz, Fmoc), 7.47 (2H, d, J = 7.7 Hz, PNB aryl), 7.39 (2H, dd, J = 8.0, 6.5 Hz, Fmoc), 7.30 (2H, dd, J = 7.5, 7.0 Hz, Fmoc), 5.44 (1H, d, J = 8.0 Hz, –NH-), 5.18 (2H, pd, J = 14.0 Hz, benzyl), 4.38 (2H, m, Fmoc-CH_2-), 4.33 (1H, m, C_αH), 4.19 (1H, t, J = 6.7 Hz, Fmoc-CH-), 2.50 (2H, m, Glu), 2.25 (1H, m, Glu), 1.97 (1H, m, Glu), 1.47 (9H, s, tBu).

Fmoc-Glu(OPNB)-OtBu (1.2 g, 2.14 mmol) was dissolved in CH_2Cl_2 (25 mL) and TFA/thioanisole (8 mL/0.5 mL) was added. After stirring at 25 °C for 2.5 h, the solvent was

removed in vacuo. The desired Fmoc-Glu(OPNB)-OH was purified by silica gel chromatography (3:1 hexane:ethyl acetate, then 1:1 hexane:ethyl acetate containing 2% acetic acid), providing a glassy, colorless solid (1.05 g, 97%): TLC (2:1 hexane:ethyl acetate) R_f Fmoc-Glu(OPNB)-OtBu 0.48, Fmoc-Glu(OPNB)-OH 0.13; ^{1}H NMR (DMSO-d_6) δ 12.65 (1H, brs, acid), 8.18 (2H, d, J = 8.5 Hz, PNB aryl), 7.97 (2H, d, J = 8.0 Hz, Fmoc), 7.69 (2H, d, J = 7.5 Hz, Fmoc), 7.65 (1H, d, J = 8.0 Hz, -NH-), 7.60 (2H, d, J = 8.5 Hz, PNB aryl), 7.39 (2H, dd, J = 8.0, 6.5 Hz, Fmoc), 7.30 (2H, dd, J = 8.0, 6.5 Hz, Fmoc), 5.22 (2H, s, benzyl), 4.26 (2H, d, J = 7.0 Hz, Fmoc-CH_2-), 4.20 (1H, m, Fmoc-CH-), 3.97 (1H, m, C_αH), 2.04 (2H, m, Glu), 1.84 (2H, m, Glu).

N-(N^α-4-Benzoylbenzoyl-L-biocytinoyl)-N′-Phe-Pro-Arg CMK (BBB-FPR CMK). To a solution of N^α-(4-benzoylbenzoyl)-L-biocytin (80 mg, 0.15 mmol), HCl-Phe-Pro-Arg CMK (25 mg, 0.05 mmol), and diisopropylethylamine (20 μL, 0.11 mmol) in DMF (0.5 mL) was added DCC (31 mg, 0.15 mmol) in one portion. The mixture was stirred at 0 °C for 2 h, then at 25 °C overnight. The reaction was filtered and concentrated to give a white solid. The material was purified by silica gel chromatography (20:1 CH_2Cl_2:CH_3-OH containing 0.01% AcOH) to afford amide BBB-FPR CMK as a white crystalline solid in 45% yield: TLC (20:1 CH_2Cl_2:CH_3OH containing 0.01% AcOH) R_f = 0.3; ^{1}H NMR (DMSO-d_6) δ 9.77 (1H, s), 8.41 (1H, s), 8.00 (2H, t, J = 6.5 Hz), 7.84−7.62 (7H, m), 7.55 (2H, t, J = 6.5 Hz), 7.24−7.06 (7H, m), 6.77 (1H, s), 6.67 (1H, s), 6.40 (1H, s), 6.34 (1H, s), 5.73 (H, s), 4.42 (1H, brs), 4.34−4.24 (3H, m), 4.12−4.08 (2H, m), 3.30 (2H, brs), 3.26 (2H, brs), 3.08−2.75 (5H, m), 2.56−2.52 (4H, m), 2.42−2.38 (2H, m), 2.08−2.00 (2H, m), 1.60−1.20 (19H, m), 0.91 (2H, d, J = 6.5 Hz).

N-(N^α-4-Benzoylbenzoyl-L-Biocytinoyl)-N′-(E-64)-1,6-diaminohexane (BBB-E-64). To a solution of *N*-(N^α-4-benzoylbenzoyl-L-biocytinoyl)-*N*′-(boc)-1,6-diaminohexane (10 mg, 13 μmol) in 1,4-dioxane (300 μL) was added 4 M HCl in 1,4-dioxane (1.5 mL) at 0 °C. The mixture was stirred at 0 °C for an additional 10 min, then at 25 °C for 1 h. The solution was concentrated, and the white solid dried in vacuo overnight. After the residue was dissolved in DMF (300 μL), diisopropylethylamine (50 μL, 0.29 mmol) and E-64 (8 mg, 22.4 μmol) in DMF:H_2O (1:2, 600 μL) were added sequentially. After the solution was cooled to 0 °C, DCC (10 mg, 48.5 μmol) was added. The mixture was stirred at 0 °C for 2 h, then at 25 °C overnight. The reaction was filtered and concentrated to give a white solid. The material was purified by silica gel chromatography (1:1 CH_2Cl_2:CH_3OH containing 1% AcOH) to afford amide BBB-E-64 as a white crystalline solid in 22% yield: TLC (1:1 CH_2Cl_2:CH_3OH containing 1% AcOH) R_f = 0.74; ^{1}H NMR (DMSO-d_6) δ 9.39 (1H, brs), 8.80 (2H, brs), 8.52 (1H, brs), 8.26 (2H, brs), 8.14 (2H, brs), 8.09−8.02 (4H, m), 7.82 (2H, brs), 7.80−7.64 (5H, m), 7.59−7.51 (2H, m), 6.41 (1H, s), 6.34 (1H, s), 4.39−4.31 (1H, m), 4.30−4.22 (2H, m), 4.10−4.06 (1H, m), 3.09−2.96 (4H, m), 2.81−2.74 (2H, m), 2.00 (2H, t, J = 6.9 Hz), 1.81−1.62 (28H, m), 1.48−1.32 (12H, m), 1.28−1.18 (6H, m), 0.86 (3H, d, J = 6.0 Hz), 0.81 (3H, d, J = 6.0 Hz); FABMS 1018 ($[M+H]^+$).

RESULTS

Isolation of Proteolytic Activity. After its GTP extraction from isolated ROS, a sample of transducin heterotrimer was directly placed on a blue sepharose column which allows for the separation of T_α and $T_{\beta\gamma}$. These initial attempts at purifying transducin α and $\beta\gamma$ subunits were complicated by the rapid decomposition of $T_{\beta\gamma}$ over 1−7 days. This degradation was characterized by the formation of a less hydrophobic peak in the HPLC chromatogram of T_γ (vide infra). The electrospray ionization mass spectrum of this peak was determined (Figure 1), and the observed mass was found to correlate to T_γ which has had the carboxyl terminal glycylfarnesylcysteine methyl ester (GFCM) moiety removed (observed mass, 7953.9 ± 0.8 Da, theoretical mass = 7952.5 Da). It was evident that some extraneous factor was present in the protein samples which resulted in this cleavage of T_γ. This decomposition was eliminated when hexylagarose chromatography, a method known to further purify the crude transducin preparation (Fung et al., 1981), was employed (Figure 2). When purified $T_{\beta\gamma}$ was treated with the protein fractions that had been removed during hexylagarose chromatography, this degradation, which had been observed earlier, could be reproduced.

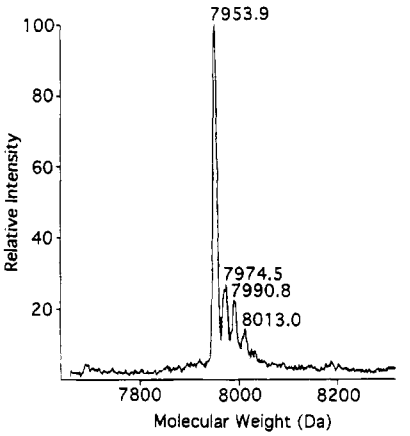


FIGURE 1: Deconvoluted electrospray ionization mass spectrum of proteolyzed T_γ. The product of the endoprotease cleavage, as seen in Figure 3, was collected and the mass was determined by electrospray ionization mass spectrometry. This peak was similarly characterized by matrix-assisted laser desorption mass spectrometry, providing the same molecular weight (theoretical mass, T_γ minus GFCM: 7952.5 Da). A peak corresponding to T_γ minus FCM (theoretical mass: 8009.5 Da) is not apparent.

Proteolytic Processing of $T_{\beta\gamma}$. Freshly prepared $T_{\beta\gamma}$ was treated with a partially purified preparation of the proteolytic activity, and the results of the subsequent processing of $T_{\beta\gamma}$ are shown in Figure 3. As can be seen here, a heat-sensitive proteolytic cleavage occurs to generate a new less hydrophobic fragment of T_γ. This fragment has been observed previously during the isolation of transducin (Ohguro et al., 1991; Parish & Rando, 1994; Bigay et al., 1994). The activity of the proteolyzed $T_{\beta\gamma}$ was next determined by measuring its ability to catalyze GTP-γ-S uptake by T_α in the presence of photolytically activated rhodopsin. These studies were performed with detergent−solubilized and purified rhodopsin. As seen in Figure 4, freshly prepared proteolyzed $T_{\beta\gamma}$ is inert with respect to catalyzing the aforementioned exchange reaction.

A time course for the proteolysis reaction was carried out next. As shown in Figure 5, proteolysis proceeds slowly, but close to completion over a 16 h period. Even with the relatively crude preparation of protease used here, no further processing of T_γ was observed over longer time periods. A pH versus rate profile for the proteolysis was also performed

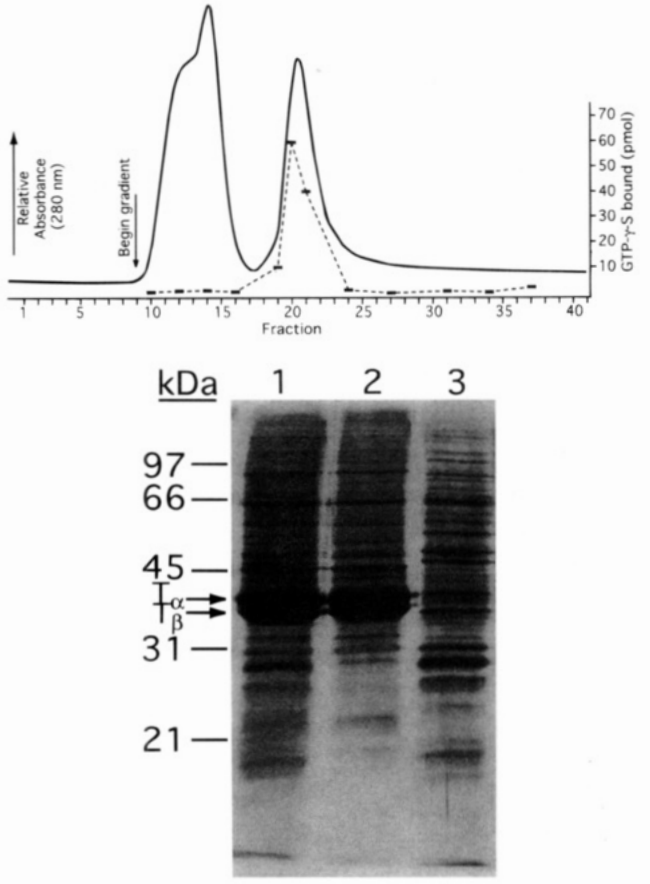


FIGURE 2: Purification of crude transducin by hexylagarose chromatography: (A, top) A crude preparation of transducin heterotrimer ($T_{\alpha\beta\gamma}$) was purified by hexylagarose chromatography as described in Materials and Methods. The UV absorbance (280 nm, solid line) and GTP-γ-S binding activity (dashed line) of each fraction is noted. (B, bottom) Silver-stained SDS−PAGE gel (12.5%) of fractions containing GTP-γ-S binding activity (fractions 18−28) and of those fractions with UV absorbance but without GTP-γ-S binding activity (fractions 10−16). *Lane 1*, crude transducin prior to hexylagarose purification; *lane 2*, fractions 18−28 (transducin); *lane 3*, fractions 10−16 (endoprotease). While the position of T_α and T_β are noted in this figure, T_γ runs at the dye front and is not stained well by silver stain.

(Figure 6). As seen here, a broad maximum with a peak of approximately 6 is observed. This curve can be fit to a two pK_a model using the BELL-FORTRAN program developed by Cleland (1979). The generated pK_a values are 4.4 ± 0.1 and 7.7 ± 0.1, respectively.

Characterization of the Products of Proteolysis. The proteolytic cleavage observed here could occur either processively, producing G and FCM, or endoproteolytically, producing GFCM. Experiments were carried out to distinguish between these two possibilities. $T_{\beta\gamma}$ was first methylated with [^{3}H]-CH_3-AdoMet at its farnesylated cysteine residue using crude isoprenylated protein methyltransferase (Gilbert et al., 1992). The labeled $T_{\beta\gamma}$ was treated with the proteolytic activity, and the resultant proteolyzed material was analyzed by HPLC. The issue under consideration here is whether proteolysis generates radiolabeled FCM or radiolabeled GFCM. Unlabeled FCM and GFCM were independently synthesized and could be readily separated by HPLC analysis (Figure 7). The unlabeled FCM and GFCM were added to the proteolytic digest before HPLC analysis. The HPLC radiochromatogram (Figure 7) clearly shows that

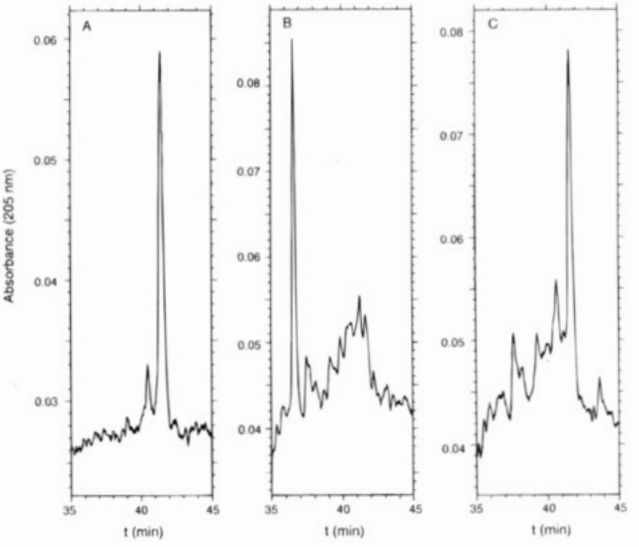


FIGURE 3: Heat sensitivity of the proteolysis of T_γ. A partially purified proteolytic activity was examined by incubation with $T_{\beta\gamma}$-OCH_3 in buffer A containing 100 mM NaCl and 5 mM $MgCl_2$. HPLC analysis I, described in Materials and Methods, was carried out to follow the extent of proteolysis: (A) $T_{\beta\gamma}$-OCH_3 (2.54 μg) was left at 25 °C for 16 h as a control; (B) the protein solution (1.3 μg) containing proteolytic activity was incubated with $T_{\beta\gamma}$-OCH_3 (2.54 μg) at 25 °C for 16 h; and (C) the protein solution (1.3 μg protein) containing proteolytic activity was heated at 95 °C for 5 min and then incubated with $T_{\beta\gamma}$-OCH_3 at 25 °C for 16 h.

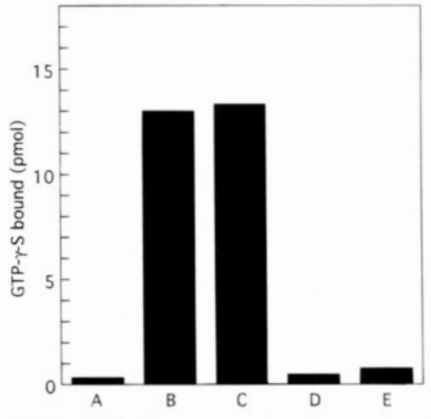


FIGURE 4: GTP-γ-S binding assay of endoproteolyzed $T_{\beta\gamma}$. (A) T_α only; (B) T_α plus untreated $T_{\beta\gamma}$; (C) T_α plus control $T_{\beta\gamma}$, left at 25 °C for 16 h; (D) T_α plus $T_{\beta\gamma}$, cleaved by endoprotease and treated with blue sepharose CL-6B; (E) T_α plus $T_{\beta\gamma}$, cleaved by endoprotease. Each assay was performed in duplicate at 0 °C. Final concentrations in assay: T_α, 0.5 μM; $T_{\beta\gamma}$, 0.3 μM; detergent-solubilized rhodopsin, 2 μM; GTP-γ-S, 10 μM, 4700 cpm pmol^{-1}. Duplicate points in all assays were within 5% of each other.

cleavage has occurred endoproteolytically, liberating GFCM. Therefore, the observed cleavage involves endoproteolytic hydrolysis.

A second issue of interest with respect to the nature of cleavage is whether or not the endoprotease distinguishes between methylated and unmethylated $T_{\beta\gamma}$. Methylated and unmethylated $T_{\beta\gamma}$ were prepared as before (Parish & Rando, 1994) and subjected to the proteolytic activity. Methylated $T_{\beta\gamma}$ is processed close to completion over 16 h (Figure 5). As shown in Figure 8, unmethylated $T_{\beta\gamma}$ was proteolytically processed to approximately the same extent over 16 h. Therefore, the state of $T_{\beta\gamma}$ methylation does not appear to be an important determinant of $T_{\beta\gamma}$ degradation.

Further structure−activity studies on the endoprotease are not feasible with $T_{\beta\gamma}$, and thus synthetic peptides were

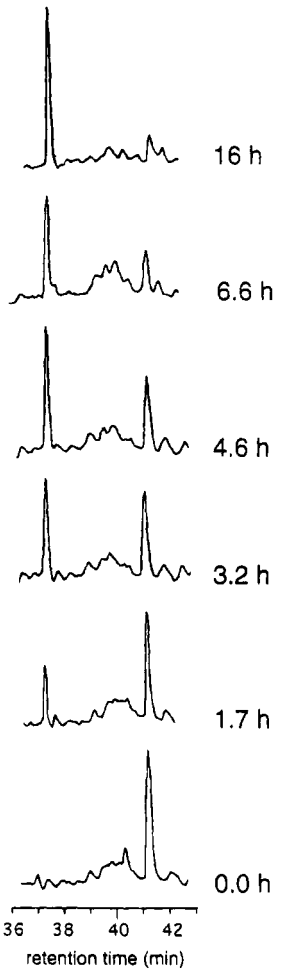


FIGURE 5: Time course of $T_{\beta\gamma}$-OCH_3 proteolysis. $T_{\beta\gamma}$-OCH_3 (120 μL, 0.20 mg mL^{-1}) in buffer A containing 100 mM NaCl and 5 mM $MgCl_2$ was incubated with crude protease (120 μL, 0.068 mg mL^{-1}) at 25 °C. At each time point, an aliquot (40 μL) was analyzed by HPLC analysis I, as described in Materials and Methods. The examined time points are 0, 1.7, 3.2, 4.6, 6.6, and 16 h.

evaluated as possible substrates for the enzyme. Two peptides (methylated and unmethylated) were synthesized [Ac-LKGGC(farnesyl)-OX and Ac-NPFKELKGGC-(farnesyl)-OX; X = H and CH_3], sequences that correspond to the carboxyl terminus of T_γ, and were tested as substrates for the endoprotease. Unfortunately, significant formation of GFCM was not observed when treating these peptides with the proteolytic activity. In one case [Ac-NPFKELKG-GC(farnesyl)-OH], degradation of the peptide occurred when the crude endoprotease preparation was used, but this processing did not correspond to endoprotease cleavage and probably results from the interaction of the peptide with another component of the crude protein preparation.

Group-Specific Inhibitors of the Endoprotease. Group-specific protease inhibitors were used to characterize further the endoprotease. Figure 9 shows results from a study in which the endoprotease was incubated with a series of protease inhibitors directed at different classes of proteases. The best inhibitors proved to be E-64, an irreversible thiol protease inhibitor (Hanada et al., 1978; Rich, 1986), leupeptin, a reversible competitive serine and thiol protease inhibitor (Umezawa, 1976), and soybean trypsin inhibitor (Birk, 1976). These experiments were performed without removing the inhibitors during the enzyme assays; thus

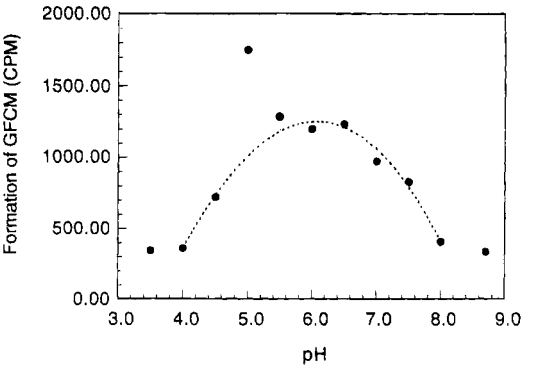


FIGURE 6: A pH versus proteolytic activity profile. The protease activity was examined by using $T_{\beta\gamma}$-[^{3}H]-OCH_3 (0.95 mg mL^{-1}, 2.4 mCi $mmol^{-1}$) as a substrate. For each pH point, $T_{\beta\gamma}$-[^{3}H]-OCH_3 (15 μg) was incubated with endoprotease (4.2 μg) in the buffers (100 mM) over a range of pH. All incubations were carried out at 25 °C for 16 h. The proteolytic process was monitored by following the formation of a radiolabeled proteolytic product (GFC-[^{3}H]-M) using HPLC analysis II, as described in Materials and Methods. The proteolytic activity has been quantitatively measured in this manner in the following buffers (final concentration, final pH): sodium citrate (20 mM, pH 3.5), sodium acetate (20 mM, pH 4.0, 4.5, 5.0, and 5.5), sodium phosphate (20 mM, pH 6.0 and 6.5), and Tris·HCl (20 mM, pH 7.0, 7.5, 8.0, and 8.7). A similar pH-profile was observed when analyzing nonradioactively labeled $T_{\beta\gamma}$-OCH_3 by HPLC analysis I, as described in Materials and Methods (data not shown).

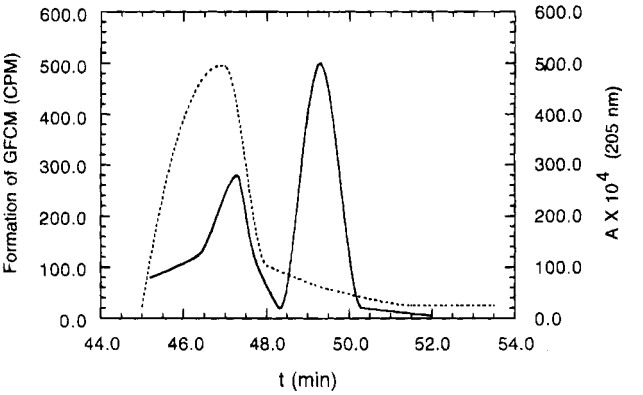


FIGURE 7: Identification of GFC-[^{3}H]-M from $T_{\beta\gamma}$-[^{3}H]-OCH_3 proteolysis. Solid line: a complete separation of synthetic, nonradioactive GFCM and FCM with retention times of 47 and 49 min, respectively, was accomplished by HPLC Analysis III, as described in Materials and Methods. This result provided an internal control for the characterization of the radiolabeled proteolytic product. Dotted line: radioactivity trace of the proteolytic product. $T_{\beta\gamma}$-[^{3}H]-OCH_3 (1.78 nmol, 0.51 mCi $mmol^{-1}$) was incubated with the protease (39 μg) at 25 °C for 16 h. Each fraction (0.4 mL) was collected and counted in Hydrofluor scintillation fluid (10 mL).

reversible and irreversible inhibition are not distinguished. Upon dialysis of the inhibited samples, it appeared that only the observed inhibition by E-64 was completely irreversible.

As shown above, both E-64 and leupeptin inhibit the enzyme. The aforementioned inhibitors share the common structural feature of possessing a terminal guanidinium moiety. Two potential histochemically active derivatives of these inhibitors were then prepared. A biotin-coupled inhibitor of BB-FPR CMK was prepared (Scheme 1), as was a biotin derivative of E-64 (Scheme 2). BB-FPR CMK, containing a benzoylbenzoyl group, was initially designed as a photoaffinity labeling reagent for identifying unknown proteases (Gilbert & Rando, 1995). Both analogues showed

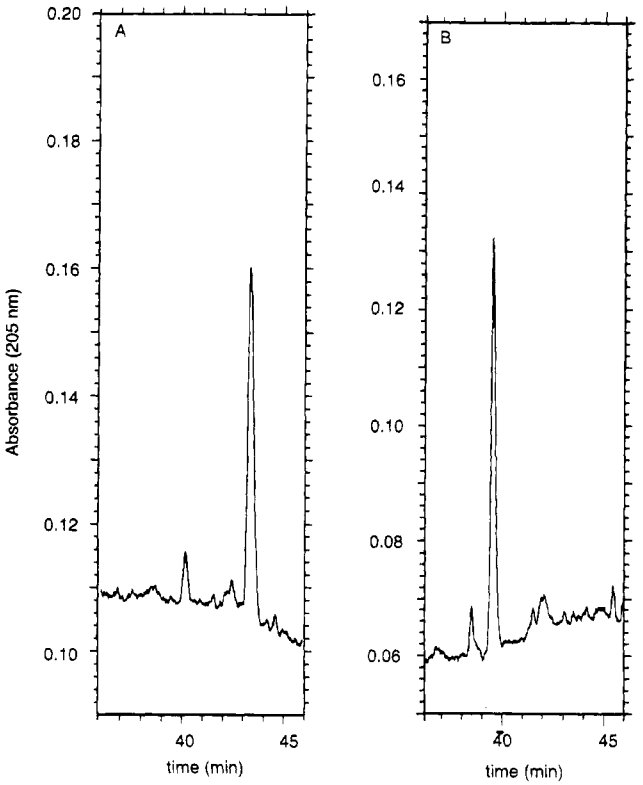


FIGURE 8: Proteolysis of unmethylated transducin γ-subunit (T_γ-OH). The proteolytic activity was monitored by HPLC analysis I, as described in Materials and Methods. (A) As a control, $T_{\beta\gamma}$-OH was left at 25 °C for 16 h in the absence of the protease. A UV peak with retention time of 43.2 min was identified as T_γ-OH. (B) $T_{\beta\gamma}$-OH (4 μg) was incubated with the protease (3 μg) at 25 °C for 16 h, and a peak at 39.6 min was identified as the cleaved T_γ fragment (minus GFCM). The product of unmethylated T_γ processing was identified by correlating it with the HPLC of methylated T_γ processing. It should be noted that the processing of a mixture of methylated and unmethylated T_γ resulted in only one product peak by HPLC, which was identified by mass spectrometry as the cleaved T_γ fragment.

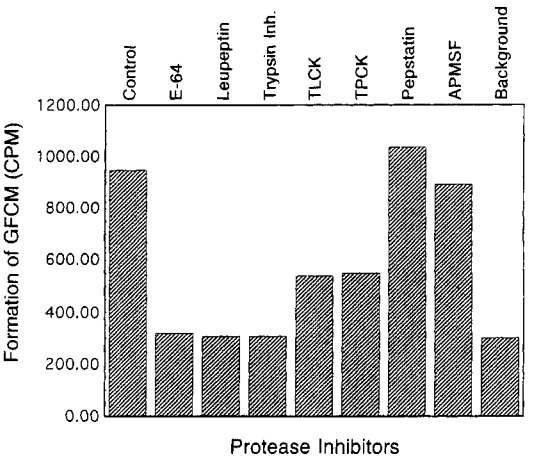


FIGURE 9: Protease inhibitor screening. A series of commercially available protease inhibitors have been examined for their ability to inhibit this endoprotease. The crude endoprotease (5.8 μg) in MOPS buffer (buffer B containing approximately 30 mM NaCl) was preincubated with a protease inhibitor (the concentration of each inhibitor is indicated below) at 25 °C for 20 min. The mixture was further incubated with $T_{\beta\gamma}$-[^{3}H]-OCH_3 (0.95 nmol, 2.4 mCi mmol^{-1}) at 25 °C for 16 h and then analyzed by HPLC analysis II as described in Materials and Methods. The fractions containing GFC-[^{3}H]-M were collected and counted. Examined inhibitors (final concentration) are E-64 (1 μM), leupeptin (1 μM), trypsin inhibitor (100 μg mL^{-1}), TLCK (130 μM), TPCK (250 μM), pepstatin (1 μM), and APMSF (20 μM). In addition, ebelactone B, a serine protease inhibitor, was also tested, but no inhibition was observed (data not shown).

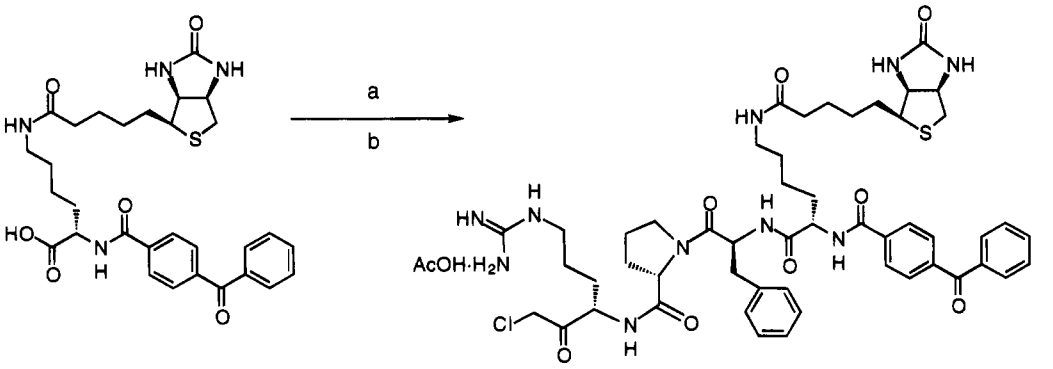
inhibition activity similar to those of their corresponding nonbiotinylated inhibitors (data not shown). Upon incubation of the crude enzyme with BBB-FPR CMK, a single protein of MW ~35 kDa was labeled, as revealed by an avidin–horseradish peroxidase (avidin–HRP) chemiluminescent detection system (Figure 10A). The labeled band is eliminated by preincubation with E-64, demonstrating specificity of action. In a second set of experiments, the crude protease was labeled with the biotinylated E-64 probe (BBB-E-64) and detected by the streptavidin–HRP system alluded to above (Figure 10B). Again, a prominent band of MW ~35 kDa was labeled. With this inhibitor, however, other less prominent bands were labeled as well. Further studies were carried out to eliminate the possibility that one of the other labeled bands could be of interest. As shown in Figure 10B, lanes 1–4, pretreatment of the endoprotease preparation with E-64 abolished labeling of the 35 kDa band, as did pretreatment with trypsin inhibitor. Moreover, pepstatin, which does not block endoprotease processing of $T_{\beta\gamma}$, did not block the labeling of the 35 kDa band. Therefore, the 35 kDa band can be taken as a candidate protein for the endoprotease.

DISCUSSION

While the enzymology of isoprenylated/methylated protein biosynthesis is well understood, nothing is known about how these proteins are degraded. The $\beta\gamma$ subunit of retinal transducin is partially found in a truncated form, having lost the terminal GFCM of T_γ (Ohguro et al., 1991; Parish & Rando, 1994; Bigay et al., 1994). This finding suggests that the truncated form may be an initial step in the degradative pathway for G proteins. It is interesting to note that one laboratory has observed a heterogeneous truncated $T_{\beta\gamma}$, lacking either FCM or GFCM at the carboxyl terminus of T_γ (Ohguro et al., 1991). As shown here, a soluble endoproteolytic activity is found in ROS which is capable of specifically cleaving T_γ to liberate the dipeptide GFCM. It is likely that the endoproteolytic activity uncovered here is the one responsible for the generation of the truncated $T_{\beta\gamma}$ found in ROS. Once the proteolytic activity has been removed, very little or no cleaved $T_{\beta\gamma}$ is isolated during the purification of transducin, as performed here. The proteolyzed $T_{\beta\gamma}$, which is generated prior to removal of the proteolytic activity, does not bind to membranes (Ohguro et al., 1991; Bigay et al., 1994) and is removed during extensive washing of the isolated ROS used in holotransducin preparation.

The fact that the enzyme does not appear to accept farnesylated peptides patterned after the C-terminus of T_γ as substrates makes it difficult to address structure–activity requirements for the enzyme. However, it was possible to demonstrate that the endoprotease is capable of hydrolyzing both the methylated and unmethylated forms of $T_{\beta\gamma}$. This result is perhaps a bit unusual in that proteases operating near a carboxyl terminus of protein would be expected to

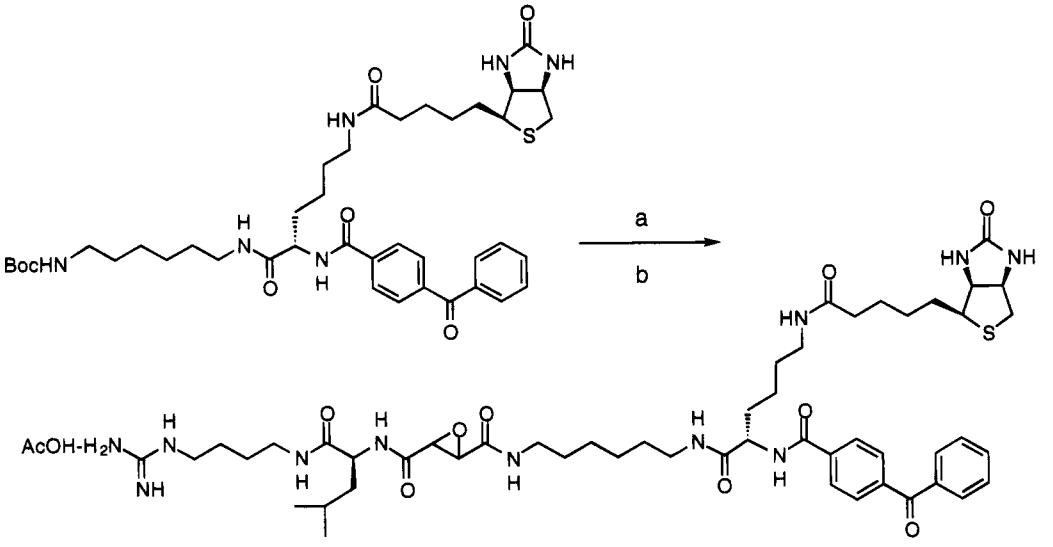
Scheme 1[a]



BBB-FPR CMK

[a] Reagents: (a) HCl, dioxane; (b) HCl-FPR CMK, Hünig's base, DCC, DMF:H_2O.

Scheme 2[a]



BBB-E-64

[a] Reagents: (a) HCl, dioxane; (b) E-64, Hünig's base, DCC, DMF:H_2O.

recognize a free carboxyl group. However, if the particular endoprotease under discussion here is designed to be involved in the global turnover of $\beta\gamma$ subunits, then it would not be surprising if the enzyme did not differentiate between methylated and unmethylated $T_{\beta\gamma}$.

How the dynamic activity of G proteins is regulated is not well understood. It is possible that in certain circumstances reversible methylation could play a role. Certainly in the case of retinal transducin, methylation of $T_{\beta\gamma}$ appears only to play a marginal role in influencing the activity of this protein (Parish & Rando, 1994; Fukada et al., 1994). The sluggish nature of the endoproteolytic reaction described here, and the fact that it is irreversible, would appear to eliminate this reaction from any further consideration as being of any regulatory significance. The most likely role for the endoprotease is probably related to protein turnover. This initial proteolytic step to remove the isoprenylated dipeptide readies the truncated, biochemically inactive $T_{\beta\gamma}$ for further proteolytic degradation.

It is interesting to note that the use of the endoproteolytic activity freshly to prepare de-isoprenylated/methylated $T_{\beta\gamma}$ confirms and strengthens the view (Ohguro et al., 1991) that isoprenylation is essential for the activity of transducin. The case is made here with rhodopsin solubilized in detergent. The role of the farnesyl moiety here may be to enhance the membrane (or micelle) affinity of $T_{\beta\gamma}$. No clear evidence has yet been published which would suggest that the farnesylated and methylated cysteine moiety of $T_{\beta\gamma}$ mediates protein−protein interactions.

Inhibitor studies on the crude endoprotease reveal one excellent irreversible inhibitor candidate (E-64) and two moderately good inhibitors (leupeptin and soybean trypsin inhibitor). Both E-64 and leupeptin contain positively charged guanidinium moieties. Given that the C-terminus of transducin ends in LKGGC (Pérez-Sala et al., 1991; Fukada et al., 1990), it is not difficult to understand why these positively charged inhibitors might be effective against the endoprotease. E-64 is exclusively a thiol protease inhibitor whereas leupeptin inhibits both serine and thiol proteases (Umezawa, 1976). Trypsin inhibitor is considered to be a trypsin-like serine protease inhibitor (Laskowski & Laskowski, 1954). Of course, there is much overlap in the reactivities of the various "group-specific" enzyme inhibitors, so that the results obtained here are not too surprising. It seems more likely that this endoprotease is a thiol protease rather than a serine protease. The reason is not only that E-64 is such a powerful inhibitor of the enzyme but also that the pH versus rate profile is reminiscent of thiol proteases

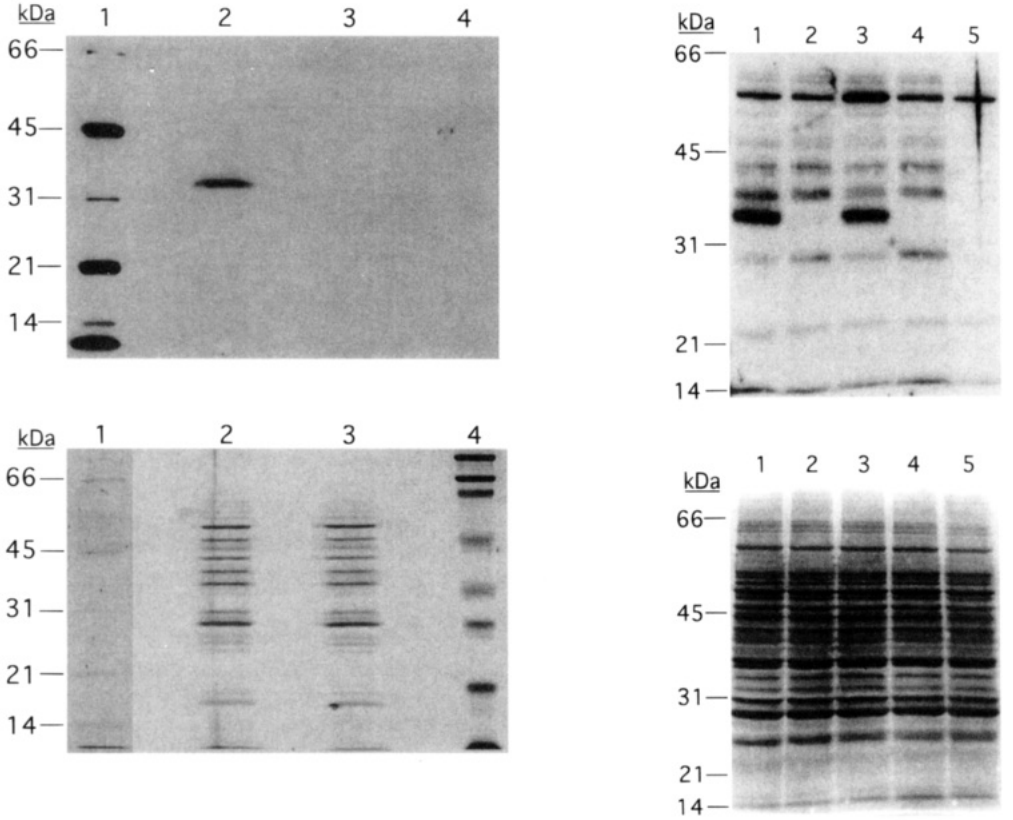


FIGURE 10: Label of the endoprotease with biotinylated probes. The crude protein solution obtained from the purification, as described in Materials and Methods, was incubated with or without a protease inhibitor and then with a biotinylated probe, processively. All these incubations were carried out in 100 mM sodium acetate (pH 5.5) at 25 °C for 20 min. Following the procedure described in Materials and Methods, labeling experiments were carried out using two biotinylated probes: BBB-FPR CMK (Scheme 1) and BBB-E-64 (Scheme 2). (Left) Biotinylated molecular weight marker (lane 1), crude protease (12.4 μg) incubated with BBB-FPR CMK (16.7 μM, lane 2), crude endoprotease (12.4 μg) pretreated with E-64 (1 μM) and then incubated with BBB-FPR CMK (16.7 μM, lane 3), prestained molecular weight marker (lane 4). (Top) The polyacrylamide gel subjected to Western blot analysis (10 sec exposure). (Bottom) The gel after blotting stained with Coomassie brilliant blue G-250. (Right) Crude endoprotease (14.4 μg) incubated with BBB-E-64 (2 μM, lane 1), protein pretreated with E-64 (1 μM) and then incubated with BBB-E-64 (2 μM, lane 2), protein pretreated with pepstatin (1 μM) and the incubated with BBB-E-64 (2 μM, lane 3), protein pretreated with trypsin inhibitor (33 μg mL^{-1}) and then incubated with BBB-E-64 (2 μM, lane 4), protein only (lane 5). (Top) The polyacrylamide gel, subjected to Western blot analysis (60 min exposure). (Bottom) Gel after blotting, stained with DAIICHI silver stain II.

(Stockell & Smith, 1957; Hinkle & Kirsch, 1971), with pK_a values of 4.4 ± 0.1 and 7.7 ± 0.1. It should be further noted that it is our impression that the "instability" of transducin is probably due to the proteolysis described here. Indeed, removal of fractions containing the endoprotease activity results in $T_{\beta\gamma}$ which is stable at 4 °C over several weeks. E-64 should probably be routinely included in the buffer during the purification of retinal transducin to prevent degradation of the protein.

Not currently having a good quantitative assay for the endoprotease makes it difficult to approach further purification. However, some of the biotinylated protease inhibitors described here may be useful for identifying this protein. Both the E-64 conjugate (BBB-E-64) and the FPR CMK conjugate (BBB-FPR CMK) labeled the same approximately 35 kDa protein band. It should be possible to obtain enough of this material for sequencing, cloning, and eventual expression. It should then be possible to determine if this protein is relevant as the holo-endoprotease or as a subunit thereof.

ACKNOWLEDGMENT

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