Isolation and characterization of grancalcin, a novel 28 kDa EF-hand calcium-binding protein from human neutrophils

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A novel 28 kDa protein, which we have named 'grancalcin', has been identified in human neutrophils. The protein was isolated from the cytosol and found to be a homodimer, with an apparent molecular mass of 55 kDa on gel filtration. Polyclonal antibodies were raised to the native protein. N-Terminal sequence analysis and tryptic-peptide sequence analysis was performed. The protein exhibits sequence similarity to sorcin, a 24 kDa calcium-binding protein over-expressed in certain multi-drug-resistant cell lines. It appears to be a member of the EF-hand family of calcium-binding proteins. The association of a high proportion of this protein with the membranes and granules in the presence of physiological concentrations of calcium may indicate a role in granule-membrane fusion and degranulation.

INTRODUCTION

Phagocytic cells contain an NADPH oxidase which catalyses the reduction of molecular oxygen to superoxide and other more toxic oxygen species (Babior, 1987; Segal, 1989). This system is important for the killing of micro-organisms. Failure to produce superoxide results in a disease known as chronic granulomatous disease (CGD), a syndrome characterized by increased susceptibility to infection. CGD is genetically heterogeneous, giving rise to defects in different components of the oxidase, the end result being lack of superoxide production. To date, this heterogeneity has provided the means of identification of three components involved in the generation of superoxide. These are the membrane-associated cytochrome b_{-245} which is the terminal component of the electron-transport chain, donating electrons to oxygen, and two cytosolic components, p47-phox and p67-phox which translocate to the membrane upon cell stimulation (Segal *et al.*, 1985, 1986; Heyworth & Segal, 1986; Parkos *et al.*, 1987; Teahan *et al.*, 1987; Dinanier *et al.*, 1987; Nunoi *et al.*, 1988; Clark *et al.*, 1989). Although the complete amino acid sequences of these latter two proteins have been deduced, their functions are as yet unknown (Lomax *et al.*, 1989; Volpp *et al.*, 1989; Teahan *et al.*, 1990). One approach to the identification of further components of the oxidase is the study of the whole group of proteins which translocate from the cytosol to the membrane upon stimulation. Antibodies to membranes from cells activated with phorbol 12-myristate 13-acetate (PMA) (which stimulates both the NADPH oxidase and degranulation) were used in the present study to probe cytosol from unstimulated cells, thus identifying proteins common to both, including those which translocate. Here we focus on the identification of a novel 28 kDa protein distributed throughout the cell, which exhibits strong sequence similarity to sorcin, a calcium-binding protein which is over-expressed in certain multi-drug-resistant cell lines (Koch *et al.*, 1986; Van der Bliek *et al.*, 1986; Meyers *et al.*, 1987). We have named this protein grancalcin.

EXPERIMENTAL

Materials

PMA, *N*-formyl-Met-Leu-Phe (fMLP), the calcium ionophore

A23187, di-isopropyl fluorophosphate (DIFP) and tosyl-l-lysylchloromethane (Tos-Lys-CH₂Cl) were from Sigma. Trypsin, sequencing grade, was from Boehringer-Mannheim. H₃³²PO₄ was obtained from ICN Radiochemicals. Poly(vinylidene difluoride) (PVDF) membrane was from Millipore.

Preparation and fractionation of neutrophils

Neutrophils were prepared from leukopheresis samples of patients with chronic granulocytic leukaemia (CGL), from buffy-coat residues obtained from the South London Blood Transfusion Centre, Tooting, London S.W.17, U.K., or from venous blood obtained from normal subjects (Segal & Jones, 1980). The cells, treated with DIFP (final concn. 1 mM) were resuspended in breaking buffer [6% sucrose (w/w) in 0.6 × relaxation buffer (1 × = 100 mM-KCl/10 mM-Pipes/3 mM-NaCl/3.5 mM-MgCl₂, pH 7.3), including 27 μM-Tos-Phe-CH₂Cl] to a final concentration of 5 × 10⁸/ml and disrupted by nitrogen cavitation at 4.14 MPa (600 lbf/in²) (Borregaard *et al.*, 1983). Cytosol and membranes were then prepared by centrifugation on sucrose gradients as described previously with the exception that the gradients were prepared in 0.6 × relaxation buffer (Segal & Jones, 1980).

³²P-labelling and stimulation of cells

Neutrophils, isolated from the blood of normal subjects, were labelled with ³²P as described previously (Heyworth *et al.*, 1989). Stimulation of the cells (1 × 10⁸ per ml) was performed by the addition of PMA (0.3 μM), fMLP (1 μM) or A23187 (2 μM), and incubating at 37 °C for 3 min. Stock solutions (1 mM) of these agents were prepared in dimethyl sulphoxide and stored in aliquots at –20 °C before use. The labelled cells were disrupted and centrifuged as described above with the inclusion of a layer of 50% (w/w) sucrose to recover the granule fraction.

Electrophoresis, autoradiography and Western blotting

These procedures were performed as described previously (Teahan *et al.*, 1990). Antiserum to grancalcin was used at a dilution of 1:400 on immunoblots. A modified Western-blotting technique was employed when the blots were to be used for amino-acid-sequence determination (see below).

Abbreviations used: CGD, chronic granulomatous disease; CGL, chronic granulocytic leukaemia; PMA, phorbol 12-myristate 13-acetate; fMLP, *N*-formyl-Met-Leu-Phe; Tos-Lys-CH₂Cl, tosyl-l-lysylchloromethane ('TLCK'); DIFP, di-isopropyl fluorophosphate; PVDF, poly(vinylidene difluoride); FCA and FIA, Freund's complete and incomplete adjuvants.

Glycosylation studies

A glycan detection kit from Boehringer Mannheim was used to determine whether or not grancalcin was glycosylated.

Production of antisera

Polyclonal antisera were raised to the membrane fraction of neutrophils prepared as described above. A 1.5 mg portion of total membrane protein (1 ml), emulsified in an equal volume of Freund's complete adjuvant (FCA), was injected subcutaneously into each of two New Zealand White female rabbits. The first two booster injections were given in Freund's incomplete adjuvant (FIA) at 2-week intervals after the initial injection. Subsequent boosts were given at 8-week intervals. IgG specific for the 28 kDa protein was then prepared by incubating Western blots of non-activated cytosol with the antiserum raised to activated membranes. Cytosol (1 mg) was electrophoresed on an SDS/12.5%-polyacrylamide gel (200 mm × 160 mm × 1.5 mm) and then blotted on to nitrocellulose. The blot was blocked and then incubated with antiserum diluted 1:100 in block solution (Teahan *et al.*, 1990) overnight at room temperature, then washed as described previously and rinsed with water. Vertical strips were cut out at intervals along the blot and these were incubated with second antibody and colour development performed in the usual way. This allowed for an accurate localization of the 28 kDa band, and hence the IgG bound to it, in those sections of the blot which were not incubated with second antibody. Anti-p28 IgG was then removed from the nitrocellulose, after several wash steps, by incubation with 0.1 M-glycine, pH 2.5, for 1 min. The eluate was then diluted 10-fold with block solution and stored at 4 °C.

Polyclonal antisera to the native 28 kDa protein were raised in two Dutch Lop Cross female rabbits. Native protein (100 µg in 20 mM-Tris/HCl, pH 7.3) was emulsified in an equal volume of FCA and injected subcutaneously. The rabbits were boosted 3 weeks later by a subcutaneous injection of 40 µg of protein emulsified in FIA, followed by subsequent boosts of 10 µg of protein in FIA at 5-week intervals. High-titre antisera were present by day 45.

Column chromatography

Purification of grancalcin was performed at 4 °C using a Pharmacia f.p.l.c. system. All buffers were filtered through a 0.45 µm-pore-size millipore filter and de-gassed before use.

Amino-acid-sequence analysis

Amino-acid-sequence analysis was performed using an Applied Biosystems model 477A pulse-liquid sequencer.

Determination of N-terminal sequence

Approx. 10 µg of partially purified protein was electrophoresed on an SDS 10%-polyacrylamide mini-gel and then blotted onto PVDF membrane (Immobilon-P; Millipore) using an LKB/Pharmacia semi-dry blotting apparatus at 0.5 A (constant amperage) at 4 °C for 15 min. (LeGendre & Matsudaira, 1989). The blot was stained with Coomassie Blue and the predominant band, at 28 kDa, cut out. A section, representing approx. 50 pmol of protein was then loaded on to the sequencer.

Tryptic-peptide mapping

The native protein was reduced and alkylated, then digested with trypsin as described elsewhere (Stone *et al.*, 1989). The reaction was terminated by freezing at -20 °C. Isolation of tryptic peptides was performed at room temperature using a Hewlett-Packard HP 1090 h.p.l.c. system. Peptides were resolved

on a C-8 Applied Biosystems Aquapore RP-300 7 µm reverse-phase column (100 mm × 2.1 mm) using a gradient of acetonitrile.

CNBr cleavage

Protein (approx. 5 µg) was blotted on to PVDF membrane as described above, stained with Ponceau S (0.2%) and then cleaved with CNBr (LeGendre & Matsudaira, 1989). Peptides were recovered by acetone precipitation, run on an SDS/15%-polyacrylamide gel and blotted on to PVDF membrane for 10 min at 0.5 A. Bands stained with Ponceau S were excised and loaded directly on to the sequencer.

Calcium binding

The ability of the protein to bind calcium was tested by several methods: staining with the cationic carbocyanine dye Stains-All (Maruyama & Nonomura, 1984), ⁴⁵Ca overlay of blotted protein (Maruyama *et al.*, 1984), the Hummel and Dryer (1962) method using ⁴⁵Ca, differential migration of the calcium-loaded and calcium-depleted species on SDS/PAGE (Burgess *et al.*, 1980) and staining with Ruthenium Red (Charuk *et al.*, 1990).

RESULTS

Initial identification of grancalcin

Antibodies raised to activated membranes reacted with numerous cytosolic proteins on Western blots (Fig. 1a). When a

Fig 1. Identification of grancalcin

(a) Western blot of human neutrophil cytosol probed with antiserum raised to membranes from PMA-activated cells. The cytosol was separated on a 10%-polyacrylamide mini-gel and transfer performed at 0.8 mA/cm² for 60 min in 25 mM-Tris/192 mM-glycine/0.1% SDS/4% (v/v) methanol, pH 8.3, using an LKB 2117-250 NOVABLOT blotting apparatus (Pharmacia LKB Biotechnology, Milton Keynes, Bucks., U.K.). The blot was then stained with Ponceau S (0.2%) for 1 min to reveal protein bands, blocked for 1 h in blocking solution [0.05% (v/v) Tween 20/3% (w/v) BSA/10% (v/v) normal goat serum/1% (w/v) gelatin/0.1% (w/v) Na₂S₂O₄ in 50 mM-Tris/20 mM-NaCl (TBS), pH 7.4] and incubated for 1 h with antiserum (1:500 dilution in blocking solution) raised against membranes isolated from PMA-activated neutrophils. The blot was washed for 1 h in Tris-buffered saline containing 0.05% Tween 20, incubated for 1 h with alkaline-phosphatase labelled goat anti-rabbit IgG (1:7500 dilution in blocking solution), and then rinsed in wash solution as described above with a final rinse without Tween 20. Colour was developed using NitroBlue Tetrazolium and 5-bromo-4-chloroindol-3-yl phosphate according to the supplier's instructions (Stratagene, La Jolla, CA, U.S.A.). Grancalcin is indicated by the star (★). (b) (NH₄)₂SO₄ cuts of cytosol from cells activated with PMA were performed at 4 °C, from 15 to 40% (satn.) in 5% steps. Aliquots of each precipitate and the final 40% supernatant were electrophoresed on an SDS/10%-polyacrylamide gel and stained with Coomassie Blue. 1, molecular-mass (M) markers; 2, whole cytosol; 3, 40% supernatant; 4, 15%-satd.-(NH₄)₂SO₄ ppt.; 5, 20% ppt.; 6, 25% ppt.; 7, 30% ppt.; 8, 35% ppt.; 9, 40% ppt. Grancalcin is indicated by the stars (★).

Fig 2. Anion-exchange chromatography of the 15%-satd.-(NH₄)₂SO₄ cut of neutrophil cytosol

Main Figure: elution profile of the 15%-(NH₄)₂SO₄ cut chromatographed on a Mono Q column (10 mm × 50 mm) equilibrated in 20 mM-Tris/HCl, pH 8.0, including 27 μM-Tos-Lys-CH₂Cl. Protein was eluted with an NaCl gradient; the flow rate was 1 ml/min. Inset: SDS/10%-PAGE of 50 μl of selected column fractions. a, Low-molecular-mass (*M*) markers; b, whole cytosol; c, 15%-(NH₄)₂SO₄ precipitate, i.e. start for the mono Q column. Numbers indicate column fractions. Protein was stained with Coomassie Brilliant Blue. Grancalcin is indicated by the arrows.

comparison was made of the pattern of reactive bands present in cytosol samples from different preparations, considerable variation was noted in the intensity of a band at approx. 28 kDa. The most likely explanation for this was thought to be slight differences in the conditions of cell disruption, most importantly the prevailing calcium concentrations. Further study revealed that the protein was sensitive to calcium concentrations in the submicromolar range (Boyhan *et al.*, 1992). In view of this and the role which calcium plays in activation of the neutrophil we decided to identify this protein and attempt to establish its function.

Purification of the 28 kDa protein (grancalcin)

All steps were performed at 4 °C. Cytosol, prepared from PMA-stimulated neutrophils, which had been stored at -20 °C, was cleared of particulate material by centrifugation at 70000 *g* for 30 min. It was established from preliminary (NH₄)₂SO₄ fractionation that all of the 28 kDa protein had precipitated by 30% saturation. Finer cuts, in 5% steps from 15 to 40%, were then performed; 15%-satd. (NH₄)₂SO₄ precipitated the 28 kDa protein with minimal contamination, and although the 20 and 25% cuts also contained some 28 kDa protein, the 15% cut was used for its greater relative purity (Fig. 1b). Precipitated protein was recovered by centrifugation at 70000 *g* for 30 min at 2 °C. The pellet was washed with relaxation buffer containing Tos-Lys-CH₂Cl, and then resuspended in 20 mM-Tris/HCl, pH 8.0, containing Tos-Lys-CH₂Cl. Some material remained insoluble and was removed by centrifugation at 70000 *g* for 30 min. The supernatant was applied to a Mono Q column (10 mm × 50 mm) equilibrated in 20 mM-Tris/HCl, pH 8.0, containing Tos-Lys-CH₂Cl. Bound protein was eluted with an NaCl gradient (Fig. 2). Fractions were trichloroacetic acid-precipitated and run in duplicate on SDS/10%-polyacrylamide gels, which were then either stained with Coomassie Blue or blotted and probed with affinity-purified antibody to protein p28. Grancalcin was eluted between 0.24 and 0.28 M-NaCl and was purified to homogeneity by

Fig 3. Gel filtration of grancalcin

Fractions 14–16 from the Mono Q column run were pooled and applied to a Superdex 75 column (16 mm × 600 mm) equilibrated in 20 mM-Tris/HCl, pH 8.0, including 0.15 M-NaCl and 27 μM-Tos-Lys-CH₂Cl. The flow rate was 1 ml/min and the temperature 20 °C. Main Figure: elution profile. Inset: 10% SDS/PAGE of column fractions: 1, column sample; 2, 54; 3, 56; 4, 58; 5, 60; 7, molecular-mass (*M*) markers.

gel filtration on a Superdex 75 column (Fig. 3). The protein sometimes ran as a doublet on SDS/PAGE. At all stages of the initial purifications of grancalcin the fractions containing the protein were located by Western blotting using the IgG eluted from nitrocellulose blots as described above. Since we have no functional assay for the protein, slot blots were used to estimate the concentration of p28 in whole neutrophils and to monitor the recovery of the protein at the various stages in the isolation procedure using the pure protein as standard. Using this method we found that p28 comprises approx. 0.3% of the total cellular protein (3 μg/10⁷ cells). A typical preparation, beginning with 2.11 g of cytosolic protein, contained 3.2 mg of grancalcin, representing 0.15% of the total protein. Approx. 28% (0.92 mg) was recovered in the 15%-satd. (NH₄)₂SO₄ pellet (total protein 5.66 mg), and 70% of this (0.63 mg), representing an overall

Fig 4. Partial amino-acid-sequence determination of grancalcin

(a) Separation of peptides from the tryptic digest of grancalcin on a C-8 reverse-phase column (100 mm × 2.1 mm) using a gradient of acetonitrile. The peptides which were sequenced are numbered 1–5. (b) (i) *N*-Terminal sequence of the protein derived from blotted material and (ii) partial sequence of a CNBr fragment separated by blotting. A second fragment was recovered which gave the same sequence as the *N*-terminus.

yield of 20%, was recovered as pure p28 from the Mono Q column.

Molecular-mass estimation

The purified protein has an apparent minimum molecular mass of 28 kDa as determined by SDS/PAGE. Gel-filtration studies gave an apparent molecular mass of approx. 55 kDa, indicating that the native protein exists as a homodimer. The absence of a reducing agent did not affect the mobility of the protein on SDS/PAGE, suggesting that the two polypeptides comprising the dimer are not disulphide-bridged.

Sequence determination

N-Terminal sequence analysis gave the sequence of the first 13 residues (Fig. 4). Peptides generated by tryptic digestion of the whole protein were separated by h.p.l.c. and five peptides were sequenced (Fig. 4). Two peptides, of approx. 12 kDa and 10 kDa, were resolved by SDS/PAGE of the CNBr-cleaved protein. The 12 kDa peptide gave the same *N*-terminal sequence as the *N*-terminus of the whole protein. Searches of the EMBL and OWL databases revealed that grancalcin showed sequence similarity to sorcin, a 24 kDa protein over-expressed in multi-drug-resistant

Fig 6. Effect of calcium on the electrophoretic migration of grancalcin and other calcium-binding proteins

SDS/12%-PAGE was performed with either 1 mM- Ca^{2+} (a) or 0.1 mM-EGTA (b) in the stacking and separating gels. 1, molecular-mass (*M*) markers; 2, calmodulin; 3, parvalbumin; 4, grancalcin; 5, BSA. Protein was stained with Coomassie Blue.

Fig 7. ^{45}Ca overlay of native slot blots of grancalcin and other calcium-binding proteins

Native protein was immobilized on nitrocellulose using a slot-blot apparatus and the membrane washed and incubated with ^{45}Ca as described in the Experimental section, then autoradiographed at -70°C . A1, parvalbumin (5 μg); A2, parvalbumin (2.5 μg); A3, parvalbumin (1 μg); A4, parvalbumin (0.5 μg); A5, tropomyosin (5 μg); A6, tropomyosin, (2.5 μg); A7, tropomyosin (1 μg); A8, tropomyosin (0.5 μg); B1, calmodulin (5 μg); B2, calmodulin (2.5 μg); B3, calmodulin (1 μg); B4, calmodulin (0.5 μg); B5, BSA (5 μg); B6, BSA (2.5 μg); B7, BSA (1 μg); B8, grancalcin (5 μg); B9, grancalcin (2.5 μg); B10, grancalcin (1 μg).

cell lines (Fig. 5) (Koch *et al.*, 1986; Van der Bliek *et al.*, 1986; Meyers *et al.*, 1987).

Post-translational modifications

Membranes, cytosol and the granule fraction from ^{32}P -labelled cells were electrophoresed and autoradiographed. We found no evidence of phosphorylation of grancalcin protein in the unstimulated cell, nor did treatment of the cells with PMA, fMLP or A23187 stimulate its phosphorylation. The protein does not

Sorcin	90 MGFNEFKELWAVLNGWRQHFISFSDSRSGTVDPQELQKALTMTGFR
Grancalcin	MGFNAFKELWAALNAWKQAIGLMGYR
Sorcin	LNPQTVNSIAKRYST-SGKITFDDYIACCVKLRALTDSEFR
Grancalcin	LSPQTLTTIVKALTDFFR

Fig 5. Sequence similarity between grancalcin and sorcin

Comparison of the sequences of the internal peptides of grancalcin with similar sequences found in sorcin. Residues in grancalcin which differ from those in sorcin are in **bold type**. The partial sequence of sorcin shown above incorporates the EF-hand region of the molecule.

appear to be glycosylated, since it gave a negative result with the glycan detection kit.

Calcium binding

Evidence of calcium binding was found in the difference in migration between the calcium-loaded and calcium-depleted preparations of grancalcin on SDS/PAGE and by the binding of ^{45}Ca to slot blots of the native protein (Figs. 6 and 7).

DISCUSSION

Calcium plays an important role in the regulation of a wide variety of cellular activities. It can function as a biological second messenger by interacting with one or more members of an ever-increasing family of calcium-binding proteins (Persechini *et al.*, 1989). Here we report the isolation of a novel protein, grancalcin, from human neutrophils which appears to be a calcium-binding protein. All of the internal peptide sequences obtained from the 28 kDa protein showed sequence similarity with the E-F-hand region (including the linker regions between the helices) of sorcin, a 24 kDa EF-hand-type calcium-binding protein over-expressed in certain multi-drug-resistant cell lines (Koch *et al.*, 1986; Van der Bliek *et al.*, 1986; Meyers *et al.*, 1987). The isolation procedure was facilitated by the fact that the protein precipitated at low concentrations of $(\text{NH}_4)_2\text{SO}_4$ [virtually all of the protein had precipitated by 30 %-satd. $(\text{NH}_4)_2\text{SO}_4$] and could be identified as a prominent band in the 15- and 20 %-satd.- $(\text{NH}_4)_2\text{SO}_4$ cuts. Further purification was obtained by anion-exchange chromatography. These two steps were sometimes sufficient for complete purification of grancalcin. Gel filtration was employed when necessary as a final step. On commencement of this work we believed that p28 was a cytosolic protein. However, we have now shown that only 50 % of p28 is present in the cytosol, the remainder being attached to the membranes and granules from where it can be recovered using EDTA (Boyhan *et al.*, 1992). Higher yields of p28 may therefore be possible if the cells are disrupted in buffer containing millimolar EDTA and the cytosol recovered used as the source of protein.

The protein does not seem to be particularly labile, but sometimes migrates as a very tight doublet on SDS/PAGE. This may reflect the state of calcium loading of the protein. A shift in electrophoretic mobility was observed when the protein was electrophoresed in the presence of 1 mM-calcium rather than 0.1 mM-EDTA. This phenomenon has been noted for several other calcium-binding proteins, such as calmodulin and sorcin (Klee *et al.*, 1979; Koch *et al.*, 1986; Garrigos *et al.*, 1991).

Grancalcin does not appear to be a substrate for phosphorylation. A very small percentage of sorcin becomes phosphorylated *in vivo*, but there is no evidence that this is of relevance to its function (Meyers, 1989). We looked at the glycosylation state of grancalcin, since there is one putative glycosylation site in the sorcin molecule, although there is no evidence that it becomes glycosylated (Meyers, 1991). We found no indication that grancalcin becomes glycosylated. Another feature common to both grancalcin and sorcin is the fact that they are both homodimers (Hamada *et al.*, 1988).

Much of the protein appears to be associated with the outer surface of both primary and secondary granules and can be washed off with 2 mM-EGTA. A more detailed analysis of the effects of calcium concentration on the subcellular distribution of grancalcin is presented elsewhere and shows that, in the presence of submicromolar concentrations of calcium, most of the protein is to be found on the granules, with some also present on the plasma membrane, but virtually none in the cytosol (Boyhan *et al.*, 1992). Conversely, when the cells are disrupted in the presence of 1 mM-EGTA, grancalcin is found almost exclusively in the

cytosol. This may indicate a role in granule function. Since the role of sorcin is unknown, the sequence similarity between the two proteins does not shed any further light on the possible functions of grancalcin. Direct binding assays with ^{45}Ca have shown that sorcin is a calcium-binding protein (Koch *et al.*, 1986; Hamada *et al.*, 1988). Grancalcin was also found to bind ^{45}Ca on slot blots of the native protein. It seems likely that the function of grancalcin is regulated by changes in intracellular calcium concentrations. Such changes are known to occur upon stimulation of the cell, leading to activation of the NADPH oxidase and degranulation. This protein may serve to couple the two processes. A study of the tissue distribution of grancalcin will be important in this respect, since a restricted distribution may give further clues as to its possible function. Further work is required to characterize the calcium-binding properties and establish its role in neutrophil function.

We are grateful to the Wellcome Trust for financial support and to the staff of the Hammersmith Hospital, London, W.12, U.K. and the Royal Marsden Hospital, Sutton, Surrey, U.K. for the supply of CGL cells. We thank J. T. Curnutte and P. G. Heyworth for collaboration during the inception of this project.

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Received 24 September 1991/4 February 1992; accepted 26 February 1992