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Neutrophil specific 33 kDa protein: its Ca^{2+}- and phospholipid-dependent intracellular translocation

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A 33 kDa protein from neutrophils has been shown to associate reversibly with phosphatidylserine containing liposomes in a Ca^{2+}-dependent manner. The protein was purified from guinea pig neutrophils. Immunoblotting and cytochemical studies with polyclonal and monoclonal antibodies to the protein revealed that the protein is commonly distributed in neutrophil cytoplasm of different animal species. The protein was translocated to the plasma membrane by treatment with stimuli. Thus the 33 kDa protein is neutrophil specific and may be involved in transmembrane signaling.

Ca^{2+} dependence; Phospholipid dependence; 33 kDa protein; Neutrophil; Lipid-protein interaction; Phosphatidylserine liposome; Monoclonal antibody

1. INTRODUCTION

We have recently reported that a 33 kDa protein of guinea pig neutrophils has an ability to associate with dipalmitoylphosphatidylcholine (DPPC) liposomes containing phosphatidylserine (PS) or cardiolipin (CL) in a Ca^{2+}-dependent manner [1]. Similar association with the plasma membrane has been reported on several proteins such as Ca^{2+}- and phospholipid-dependent protein kinase (C-kinase) [2], synexin [3], calelectrin [4], etc. [5]. All these proteins, Ca^{2+} and acidic phospholipids seem to act synergistically. A rationale for Ca^{2+} and phospholipid binding might be a reversible capture and this may contribute to the regulation mechanism of transmembrane signaling within the cell. In order to clarify the characteristics of the 33 kDa protein, we tried to purify this protein by column chromatography and raised polyclonal and monoclonal antibodies to the protein. By using these antibodies, we observed the distribution of this protein in various cell types and determined its species or tissue specificity. In this report, we describe the purification method, the intracellular distribution and the stimulus-dependent translocation from cytoplasm to plasma membrane in neutrophils.

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2. MATERIALS AND METHODS

2.1. *Materials*

Balb/c mice and Hartley guinea pigs were obtained from the Shizuoka Laboratory Animal Center, Shizuoka. Durapore filters were purchased from Millipore (Bedford, MA, USA). Peroxidase and FITC conjugated with rabbit anti-mouse IgG were the products of Cappel (Cochranville, PA, USA). TSK Phenyl-5PW was obtained from LKB-Producter AB (Bromma, Sweden). Phenylmethylsulfonyl fluoride (PMSF), phospholipids, CL and FMLP were purchased from Sigma (St. Louis, MO, USA) and all other chemicals were reagent grade.

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2.2. *Purification procedure*

Guinea pig neutrophils obtained after intraperitoneal injection of 2% casein [6] were homogenized in Hepes buffer (20 mM Hepes/100 mM NaCl, pH 7.4) containing 1 mM PMSF and 1 mM EGTA with a Teflon-glass homogenizer at 4°C for 10 min. The homogenates were centrifuged at 100000 × *g* at 4°C for 60 min. The resulting clear supernatants were incubated with DPPC/CL (1:0.25 molar ratio) liposomes, which were prepared by sonication in Hepes buffer (pH 7.4), at room temperature for 10 min in the presence of 1 mM $CaCl_2$ as described [7,8]. The liposome-associated proteins were separated from non-associated proteins by discontinuous Ficoll density-gradient centrifugation (the flotation method) as described in [7,8]. Then the liposome-associated proteins were released from liposomes by treatment with 5 mM EGTA followed by centrifugation at 10000 × *g* at 4°C for 20 min. The supernatants were chromatographed on a column (8 × 75 mm) of TSK Phenyl-5PW equilibrated with 20 mM Tris/HCl (pH 7.5) containing 1 mM dithiothreitol (DTT) and 0.1 mM $CaCl_2$. Proteins were eluted with the same buffer containing 1 mM DTT and 1 mM EGTA at a flow rate of 0.5 ml/min.

2.3. *Preparation of anti-33 kDa protein antibodies*

Balb/c mice were injected subcutaneously with 50 μg of partially purified 33 kDa protein in complete Freund's adjuvant. Then injections with 50 μg protein in incomplete adjuvant were repeated six times at intervals of 2 weeks. 7 days after the last booster, sera were collected and tested for polyclonal antibodies. Moreover, 4 days after the last booster, other Balb/c mouse spleen cells immunized to partially purified 33 kDa protein and mouse myeloma cells (P3/X63, AG8U1; P3U1) were successfully fused with polyethylene glycol (PEG 4000) [9]. Hybridoma cells were selected in hypoxanthine-aminopterin-thymidine (HAT) medium for 10 days, after which the culture supernatants were tested by the enzyme-linked immunosorbent assay (ELISA) with 33 kDa protein as antigen. Positive wells were cloned in hypoxanthine-thymidine (HT) medium by limited dilution containing normal mouse macrophages as the feeder layer, and a single clone which produced the monoclonal antibody to the 33 kDa protein was obtained.

2.4. *Preparation of tissue cytosolic fraction and immunoblotting*

Various tissues of guinea pig and the neutrophils of rat, guinea pig and human were homogenized at 4°C for 10 min in Hepes buffer containing 1 mM PMSF and 1 mM EGTA. These homogenates were centrifuged at 100000 × *g* at 4°C for 60 min. The SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of these supernatants was used for immunoblotting experiments [10].

2.5. *Fluorescent staining of cells*

Indirect immunofluorescent visualization of the 33 kDa protein in bone marrow cells, intraperitoneal neutrophils and macrophages was performed essentially as described [11].

3. RESULTS AND DISCUSSION

3.1. *Purification of the 33 kDa protein*

Ca^{2+}- and acidic phospholipid-binding proteins were isolated by using the flotation method. The liposome-associated proteins were released by treatment with EGTA and applied to a column of TSK Phenyl-5PW equipped with high performance liquid chromatography (HPLC) and equilibrated with 20 mM Tris-HCl buffer (pH 7.5) containing 0.1 mM $CaCl_2$ and 1 mM DTT. Proteins that were bound hydrophobically to the phenyl column in a Ca^{2+}-dependent manner were eluted with 20 mM Tris-HCl buffer (pH 7.5) containing 1 mM EGTA and 1 mM DTT. Fractions (1 ml each) were collected at a flow rate of 0.5 ml/min. The 33 kDa protein was highly purified by this column chromatography in fraction number 21 (fig.1).

3.2. *Tissue distribution of the 33 kDa protein*

The homogenate supernatants of 6 tissues obtained from a guinea pig were applied to SDS-PAGE and used for immunoblotting with anti-33 kDa antibody. The antibody reacted strongly with the 33 kDa protein of lung and spleen but not with the ones of heart, kidney, liver and brain (fig.2). Fig.3 shows the immunohistochemical fluorescence and Giemsa stainings of guinea pig bone marrow cells. The 33 kDa protein was detected only in cytoplasm of

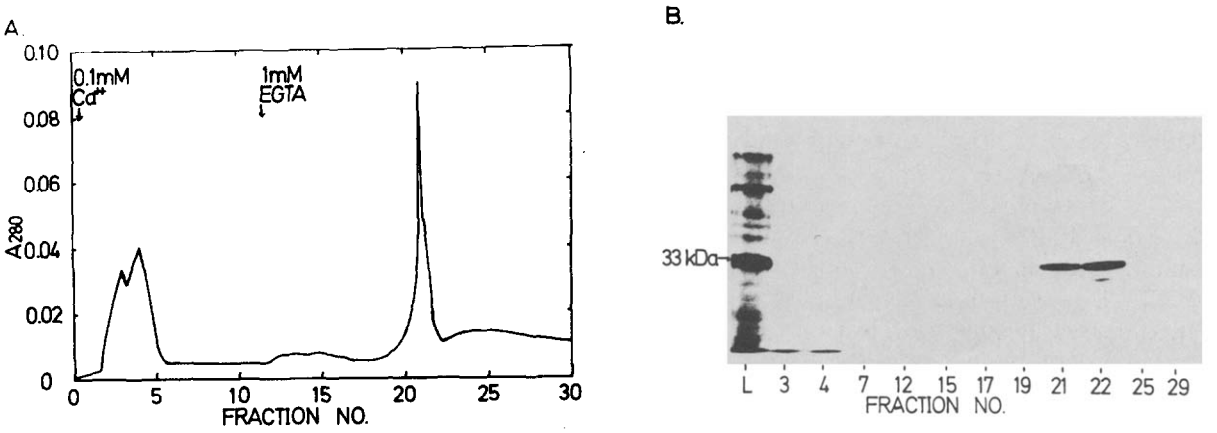

Fig.1. Ca^{2+}-dependent hydrophobic-interaction chromatography of liposome-associated protein. The Ca^{2+}- and phospholipid-binding proteins in the supernatant of guinea pig neutrophil homogenate were associated with CL-containing DPPC liposomes in the presence of 1 mM Ca^{2+} and released from the liposomes by treatment with 5 mM EGTA. The released sample, after addition of excess free Mg^{2+} and Ca^{2+}, was applied to a column (8 × 75 mm) of TSK Phenyl-5PW previously equilibrated with 20 mM Tris-HCl buffer (pH 7.5) containing 0.1 mM $CaCl_2$ and 1 mM DTT. After washing with starting buffer, the column was eluted with the same buffer containing 1 mM EGTA and 1 mM DTT. (A) Elution profile of the proteins (A_{280}). (B) Silver stained 10% SDS-PAGE of the eluted fractions: L, liposome-associated proteins.

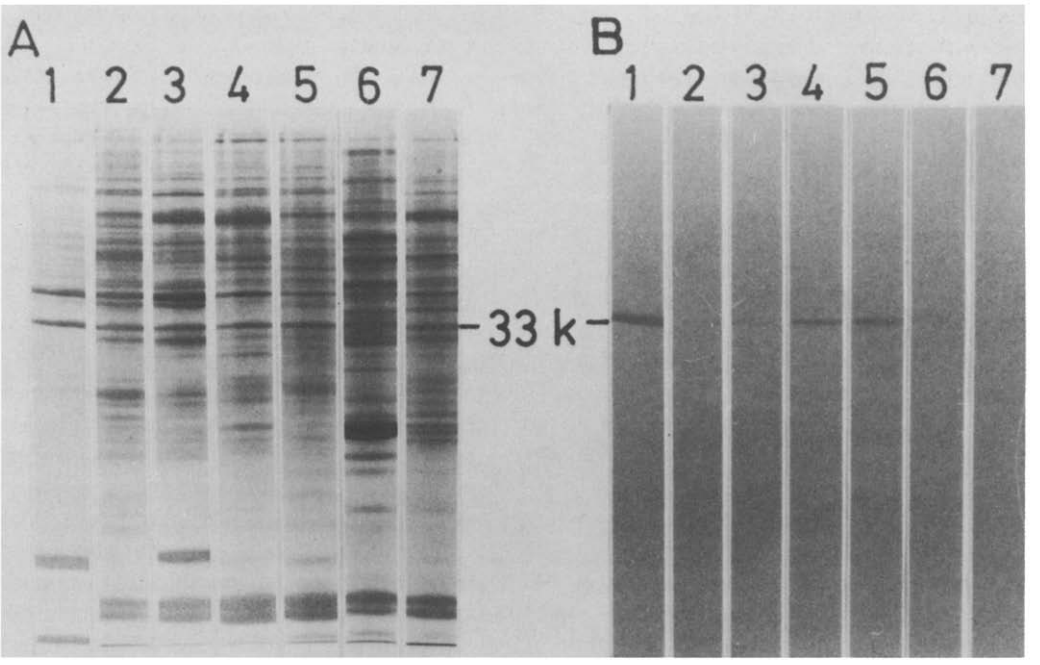

Fig.2. Immunoelectrophoretic blotting of guinea pig tissue homogenate supernatants by the polyclonal antibody to the 33 kDa protein. The homogenate supernatants of various organs obtained from guinea pigs 16 h after intraperitoneal injection of 2% casein were electrophoresed in 15% SDS-PAGE and transferred to Durapore filters. (A) Coomassie blue staining; (B) immunoblotting with mouse anti-33 kDa protein antibody. Lanes: 1, neutrophil; 2, brain; 3, heart; 4, lung; 5, spleen; 6, liver; 7, kidney.

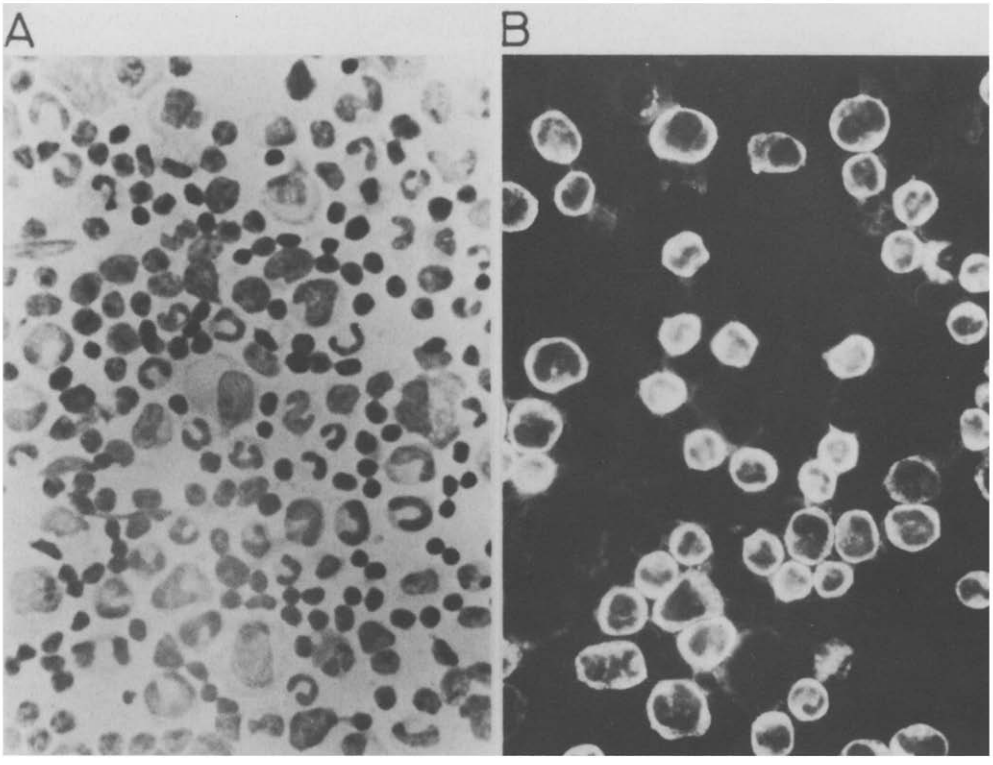

Fig.3. Indirect immunofluorescent staining of guinea pig bone marrow cells by monoclonal anti-33 kDa antibody. Bone marrow cells of guinea pigs were stained with Giemsa solution or monoclonal antibody to the 33 kDa protein. (A) Giemsa staining. (B) Distribution of the 33 kDa protein visualized by FITC labeling.

pre- and matured neutrophils. Similar immunofluorescence assays were performed on frozen sections of lung and spleen tissues. The 33 kDa protein was also detected in neutrophils distributed in these tissues (not shown). By using a similar immunofluorescence assay method, 33 kDa protein was not detected in lung or intraperitoneal macrophages. These findings suggested that the 33 kDa protein is specific to neutrophils.

3.3. *Species specificity of the 33 kDa protein*

The distribution of 33 kDa protein in homogenate supernatants of rat and human neutrophils was assayed by immunoblotting with antibody to the 33 kDa protein of guinea pig neutrophils. The antibody reacted with the 33 kDa protein of rat or human neutrophils as shown in fig.4. Therefore, the 33 kDa protein appears to be specific to neutrophils of any animal species.

3.4. *Stimulus-dependent translocation of the 33 kDa protein from cytoplasm to the plasma membrane*

As suggested in studies of other Ca^{2+}- and phospholipid-binding proteins [2–5], we detected by using the immunofluorescence technique that the 33 kDa protein had a tendency to translocate from cytoplasm to the plasma membrane in neutrophils following treatment with a stimulus such as FMLP (fig.5A,B). The precise mechanism is not known yet but it is possible that the translocation of this protein to the plasma membrane may be dependent on an increased concentration of intracellular calcium ions in neutrophils by the treatment with FMLP [12]. This idea was supported by the following experiment: the 33 kDa protein of guinea pig neutrophils was recovered in the subcellular particulate fraction in the presence of Ca^{2+} but was recovered in the soluble fraction in the presence of EGTA as shown in fig.6.

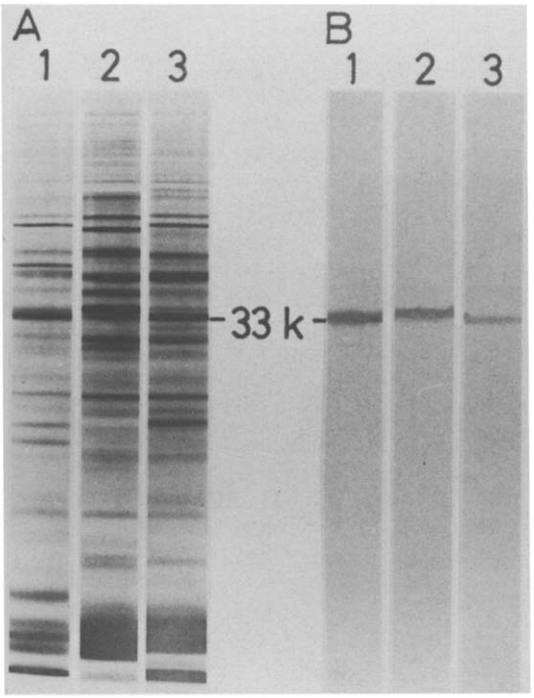

Fig.4. Immunoelectrophoretic blotting of neutrophil whole cell homogenate supernatant obtained from rat, human and guinea pig. Neutrophils of the guinea pig and the rat were obtained 16 h after intraperitoneal injection of 2% casein. Human neutrophils were isolated from whole blood by Ficoll-Conray centrifugation followed by hypotonic lysis to remove residual erythrocytes. The supernatants were electrophoresed in 15% SDS-PAGE and transferred to a Durapore filter. (A) Silver staining; (B) immunoblotting with mouse anti-33 kDa antibody. Lanes: 1, guinea pig neutrophil; 2, rat neutrophil; 3, human neutrophil.

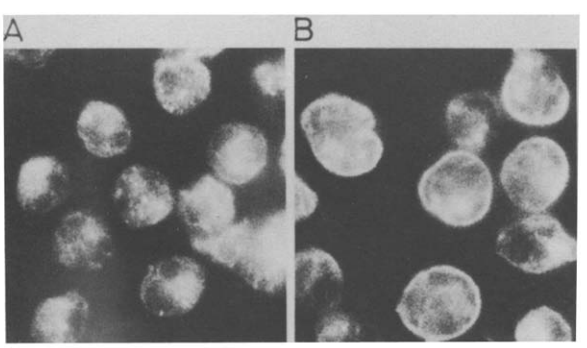

Fig.5. Translocation of 33 kDa protein from cytoplasm to the plasma membrane by treatment with FMLP. Guinea pig neutrophils were stained using the indirect immunofluorescence method. (A) Untreated; (B) treated with 10^{-6} M FMLP in the presence of 1 mM Ca^{2+} at 37°C for 10 min.

Recent reports revealed that some of the Ca^{2+}- and phospholipid-binding proteins such as C-kinase and lipocortin have important functions in transmembrane signaling within the cells, but the functions of many other proteins in this group remain unknown [5]. In preliminary experiments it was found that the 33 kDa protein in SDS-PAGE did not exhibit a Ca^{2+}-dependent mobility shift or $^{45}Ca^{2+}$ binding using the technique of $^{45}Ca^{2+}$-autoradiography of transblotted protein on Durapore filters according to Maruyama et al. [13]. A similar protein to this was also reported by McDonald et al. [14]: the 17 kDa Ca^{2+}-binding

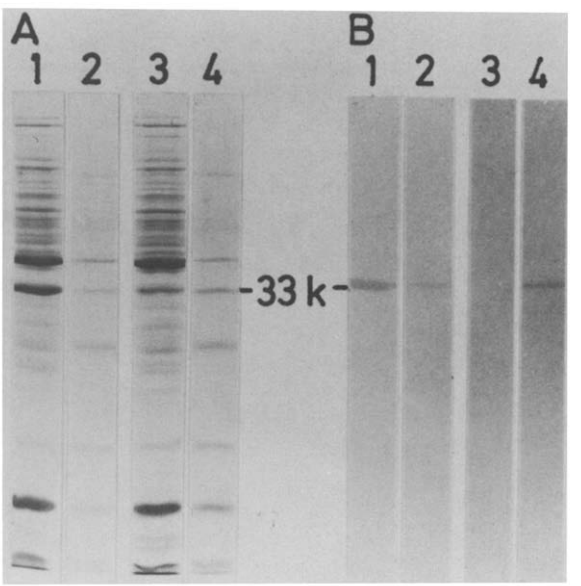

Fig.6. Distribution of 33 kDa protein in subcellular fractions in the presence or absence of Ca^{2+} in homogenizing medium. Guinea pig neutrophils were homogenized in the presence of 1 mM Ca^{2+} or 1 mM EGTA. Homogenates were centrifuged for 60 min at 100000 × *g* at 4°C and Ca^{2+}-dependent membrane binding proteins were obtained in the soluble fraction. The precipitants were treated with a buffer containing 1 mM EGTA and 0.2% Triton X-100 and centrifuged at 100000 × *g* for 60 min and Ca^{2+}-dependent membrane bound proteins were obtained in the particulate fraction. Both soluble and particulate fractions were subjected to 15% SDS-PAGE. (A) Coomassie blue staining; (B) immunoelectrophoretic blotting of 33 kDa protein. Lanes: 1 and 3, soluble fractions; 2 and 4, particulate fractions; 1 and 2, in the presence of 1 mM EGTA; 3 and 4, in the presence of 1 mM Ca^{2+}.

protein isolated from bovine brain was not detected by a Ca^{2+}-dependent electrophoretic mobility shift or by transblotting $^{45}Ca^{2+}$-autoradiography. Therefore, it seemed likely that the 33 kDa protein had a low Ca^{2+}-binding affinity. The profile of the 33 kDa protein in associating with PS-containing liposomes was the reverse of that of actin suggesting that this protein is not an actin-binding protein. At present, the biological role of the 33 kDa protein in neutrophils remains obscure but it may play an important role in the stimulation event in neutrophils. In connection with this, we are now attempting the characterization of this protein.

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REFERENCES

[1] Utsumi, K., Sato, E., Okimasu, E., Miyahara, M. and Takahashi, R. (1986) FEBS Lett. 201, 277–281.
[2] Nishizuka, Y. (1984) Nature 308, 693–698.
[3] Creutz, C.E., Pazoles, C.J. and Pollard, H.B. (1978) J. Biol. Chem. 253, 2858–2866.
[4] Sudhof, T.C., Walker, J.H. and Fritsche, U. (1985) J. Neurochem. 44, 1302–1307.
[5] Geisow, M.J. and Walker, J.H. (1986) Trends Biochem. Sci. 11, 420–423.
[6] Utsumi, K., Sugiyama, K., Miyahara, M., Naito, M., Awai, M. and Inoue, M. (1977) Cell Struct. Funct. 2, 203–209.
[7] Utsumi, K., Okimasu, E., Takehara, Y., Watanabe, S., Miyahara, M. and Morimizato, Y. (1981) FEBS Lett. 124, 257–260.
[8] Utsumi, K., Okimasu, E., Morimoto, Y.M., Nishihara, Y. and Miyahara, M. (1982) FEBS Lett. 141, 176–180.
[9] Kohler, G. and Milstein, C. (1975) Nature 256, 495–497.
[10] Towbin, H., Staehelin, T. and Gordon, J. (1974) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[11] Yoshimura, N., Hatanaka, M., Kitahara, A., Kawaguchi and Murachi, T. (1984) J. Biol. Chem. 259, 9847–9852.
[12] Horn, M. and Karnovsky, M.L. (1986) Biochem. Biophys. Res. Commun. 139, 1169–1175.
[13] Maruyama, K., Mikawa, T. and Ebashi, S. (1984) J. Biochem. 95, 511–519.
[14] McDonald, J.R. and Walsh, M.P. (1985) Biochem. J. 232, 559–567.