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A Pentapeptide from the Laminin B1 Chain Mediates Cell Adhesion and Binds the 67 000 Laminin Receptor

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Received July 22, 1987; Revised Manuscript Received September 1, 1987

ABSTRACT: Laminin promotes epithelial cell adhesion in part through a site of nine amino acids CDPGYIGSR on the B1 chain. Using smaller synthetic peptides from this sequence as well as various peptides with amino acid substitutions, we find that the minimum sequence necessary for efficient cell adhesion as well as receptor binding is YIGSR. The deletion of tyrosine or the substitution of arginine in the peptides resulted in a significant loss of activity. The presence of an amide group on the terminal arginine of either peptide increases activity significantly. YIGSR is active in promoting the adhesion of a variety of epithelial cells; however, it is inactive with chondrocytes, fibroblasts, and osteoblasts.

Laminin, the major noncollagenous glycoprotein in basement membranes (Timpl et al., 1979; Chung et al., 1979), has various biological activities (Kleinman et al., 1985; Timpl & Dziadek, 1986) including promoting epithelial cell adhesion via a 67-kilodalton (kDa) cell surface receptor (Rao et al., 1982; Malinoff & Wicha, 1983; Lesot et al., 1983). Laminin is composed of three chains designated A (M_r 400 000), B1 (M_r 210 000), and B2 (M_r 200 000) that are arranged in a cruciform-like structure (Engel et al., 1981). The laminin chains have been cloned (Barlow et al., 1984; Sasaki et al., 1987), and the B1 (Sasaki et al., 1987) and B2 chains as well as much of the A chain have been sequenced (Sasaki, unpublished results). Seven distinct structural domains occur along the B1 chain, and the B2 chain has a homologous but not identical structure. Recently, using synthetic peptides of approximately 20 amino acids corresponding to sequences in these domains and antibodies prepared to the peptides, we found that an antibody to a peptide from a region near the intersection of the three chains in domain III inhibited cell attachment to laminin while the peptide itself was inactive (Graf et al., 1987). Various peptides from adjacent sequences were synthesized and a nine amino acid peptide, CDPGYIGSR, in the B1 chain was found to be active in cell adhesion, in stimulating cell migration, and in cell surface receptor binding. Here we test smaller peptides contained within this sequence as well as various amino acid substitutions in the active peptide to determine the minimum sequence required for cell attachment and for receptor binding. Our studies

demonstrate that a five amino acid sequence, Tyr-Ile-Gly-Ser-Arg (YIGSR), is the minimal sequence which exhibits cell attachment and laminin receptor binding activity.

Experimental Procedures

Synthesis of Peptides. Peptides were synthesized with an automated Model 430A Applied Biosystems synthesizer as previously described (Graf et al., 1987). Deprotection and release of the peptides from the solid-phase support matrix were accomplished by treating the protected peptide on the resin with anhydrous HF containing 10% thioanisole or 10% anisole for 1–2 h at 0 °C. The deprotected reagents were removed by extraction with either ethyl acetate or diethyl ether, the peptide was dissolved in 10% aqueous acetic acid, and the solution was filtered to remove the resin. Synthesis of the amide form of the peptide was performed by using methylbenzhydrylamine (methyl-BHA) resin (Applied Biosystems, Inc.). After lyophilization, the composition and purity of all peptides were determined by amino acid and high-pressure liquid chromatographic (HPLC) analyses. The concentration of weighed peptides after solubilization in assay buffer was determined by amino acid analyses. Chemical modification of the arginine residue with 1,2-cyclohexanedione was performed as previously described (Patthy & Smith, 1975).

Cells and Cell Adhesion Assay. HT-1080 cells from a human fibrosarcoma (Rasheed et al., 1974) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. After the cells were grown to 80% confluency, they were washed with 0.02 M phosphate buffer, pH 7.4, without Ca^{2+} and Mg^{2+} and detached with a solution of 0.025% trypsin plus 0.025% ethylenediaminetetraacetic acid (EDTA) (GIBCO). The cells were then pelleted by low-speed centrifugation and resuspended in Eagle's minimal essential medium (EMEM) containing 0.02% bovine serum albumin (BSA). Cell attachment assays were performed as described previously (Klebe, 1974) on tissue culture plastic substrates by using 16-mm Falcon tissue culture wells incubated with various peptides or with laminin in 1 mL of EMEM containing 0.02% BSA for 1 or 2 h at 37 °C. Cells (10^5 in 0.1 mL) were added to the wells, and the solution was incubated for an additional 1 h at 37 °C in 95% air and 5% CO_2. Subsequently, the wells were rinsed 3 times with 1 mL of phosphate-buffered saline (PBS) to remove unattached cells. Attached cells were trypsinized and counted electronically (Coulter, ZBI). The attachment assays were carried out in the presence of cycloheximide (25 μg/mL) to minimize the production of endogenous attachment proteins. Each peptide was tested in duplicate dishes and each assay was repeated 6 times. The variation between samples in different assays was <15%.

Various cells were tested for their ability to attach to YIGSR-NH_2 including CHO (Chinese hamster ovary from the ATCC), MCF-7 breast carcinoma cells (from M. Lippman, NCI), G-8 muscle cells (from M. Nirenberg, NIHLB), PAM 212 epidermal cells (from J. Stanley, NCI), B16F10 melanoma cells (from I. Fidler, M. D. Anderson Hospital, Houston, TX), NG108-15 neuroblastoma x glioma hybrid cells (from M. Nirenberg, NIHLB), A431 epidermoid carcinoma cells (from I. Pastan, NCI), retrovirus-transformed chick chondrocytes (from W. Horton, NIDR), and human skin fibroblasts and bovine osteoblasts (from P. Gehron Robey, NIDR).

Elution of the Laminin Receptor with Synthetic Peptides. Detergent extracts of Engelbreth–Holm–Swarm (EHS) tumor cell membranes were used as a source of the laminin receptor (M_r 67 000). EHS tumors were maintained in mice as previously described (Orkin et al., 1977), and cell membranes were prepared from tumor tissue by a modification of the method of Cates and Holland (1978). Briefly, frozen tumor tissue (10 g/100 mL) was homogenized in 8.5% sucrose, 10 mM triethanolamine (TEA), 1 mM phenylmethanesulfonyl fluoride (PMSF), 10 mM *N*-ethylmaleimide (NEM), and 5 mM EDTA. The homogenate was placed on ice for 30 min, followed by a low-speed centrifugation (500 rpm) and subsequent collection of the membranes contained in the supernatant fraction. The pellet was resuspended in the same solution, and the homogenization, centrifugation, and collection of the supernatant fraction were repeated. The supernatant fractions were combined and filtered through gauze to remove particulate material. After collection of the membrane fraction by high-speed centrifugation, the membrane pellet was extracted for 4 h at 4 °C in 0.01 M tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl), pH 7.4, containing 1% octyl glucoside (OG) and 1 mM PMSF. The extracted material was applied to and circulated over a laminin affinity column equilibrated in 1% OG and 0.01 M Tris-HCl, pH 7.4, for 2–18 h. To remove nonspecifically bound material, the column was washed with 5 column volumes of each of the following buffers: (1) 0.01 M Tris-HCl, pH 7.4, containing 1% OG, (2) 0.01 M Tris-HCl, pH 7.4, (3) 0.5 M NaCl in 0.01 M Tris-HCl, pH 7.4, (4) 0.01 M Tris-HCl, pH 7.4, (5) a mixture of 1 mM each of glycine, serine, arginine, tyrosine, and isoleucine in 0.01 M Tris-HCl, pH 7.4, containing 1% OG, and (6) 0.01 M Tris-HCl, pH 7.4.

Subsequently, peptides at 0.6 mg/mL in 0.01 M Tris-HCl, pH 7.4, plus 1% OG were applied to the column, allowed to equilibrate for 10 min, and then eluted with 2 column volumes of 0.01 M Tris-HCl, pH 7.4, containing 1% OG. These eluates were dialyzed against H_2O, lyophilized, electrophoresed on a 7.5% polyacrylamide gel, and transferred by Western blot to nitrocellulose (Towbin et al., 1979). Antibody prepared to EHS laminin receptor in this Laboratory, and to rodent muscle laminin receptor (supplied by Dr. K. von der Mark, Max Planck Institute für Biochemie, Munich), was used at a 1:150 dilution to detect the presence of the M_r 67 000 receptor in the eluted fractions by standard immunoblot procedures. The immunoblots were photographed, and densitometric scans of the negatives were used to quantitate the relative amount of laminin receptor eluted by the various peptides.

Purification of Laminin Receptor and Preparation of Antibodies. Plasma membranes of EHS tumor were prepared, solubilized, and clarified by centrifugation at 35000*g* for 1 h as described above. Extracts were circulated over a collagen IV–Sepharose column, and unbound material was then applied to a laminin–Sepharose column. This column was exhaustively washed with 1% OG, 10 mM TEA, pH 7.4, and 1 mM PMSF and then with the same buffer plus 1 M NaCl. Bound protein was then eluted with 0.2 M glycine hydrochloride, pH 2.8. Analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) demonstrated the presence of a single polypeptide chain migrating with a M_r of 55 000 and a M_r of 67 000 after reduction of disulfide bonds, characteristic of the behavior of laminin receptor prepared from human carcinoma (Wewer et al., 1986) and from rodent muscle (Lesot et al., 1983). The EHS laminin receptor was resolved on preparative SDS gels, identified by staining with Coomassie Blue, and that portion of the gel was excised with a razor. This material was emulsified with 0.5 mL of adjuvant (Ribi Immunochemicals, Hamilton, MT) in PBS and injected in multiple subcutaneous sites in a New Zealand White rabbit

Table I: Summary of Activities of YIGSR Peptide and Its Permutations

peptide	attachment (%)	receptor binding (%)
CDPGYIGSR	64	ND
CDPGYIGSR (1,2-cyclo)	0	ND
CDPGYIGSR-NH_2	100[a]	100[b]
DPGYIGSR	50	ND
GYIGSR	52	ND
YIGSR	50	ND
YIGSR-NH_2	98	64
YIGSE	15	18
YGGGR	22	8
YIGSK	12	16
IGSR	13	15
IGSE	2	6

[a] Maximal attachment in this series of peptides was observed with 60 μg of CDPGYIGSR-NH_2 and is designated as 100%. All peptide data shown are for 60 μg. Data are the average of duplicates that did not differ by more than 15%. [b] Maximal elution of the laminin adhesion receptor from the laminin–Sepharose affinity column was observed with 0.6 mg/mL of CDPGYIGSR-NH_2 and is designated as 100%.

at 14-day intervals. Antisera showed specific reactivity with the M_r 67 000 laminin binding protein as assessed by Western blotting techniques (Towbin et al., 1979) and was shown to inhibit the attachment of mouse melanoma cells to laminin substrates (Albini et al., unpublished results), further indicating its specificity.

Results

A previous study demonstrated that a nonapeptide, CDPGYIGSR, supported the attachment of HT-1080 and CHO cells and competed with laminin for binding to the laminin receptor, suggesting that it served as a cell attachment site in laminin (Graf et al., 1987). Here (Table I) we tested variants of the nonapeptide for cell attachment activity using HT-1080 cells that attach well to laminin. Because we suspected that the terminal arginine might be important for activity, we tested the nonapeptide following modification by cyclohexanedione. Indeed this modification, specific to the guanidinium group of arginine, totally inactivated the peptide, indicating that its arginine residue was essential. Additionally, it was found that the amide form of the nonapeptide was significantly more active than the original 9-mer. Various deletions were made from the N-terminus of the peptide, which showed that the pentapeptide YIGSR retained half the activity of the nonapeptide amide while the amide form of the pentapeptide was equally active. Removal of the tyrosine residue or substitution of other amino acids for the arginine was associated with a marked loss in activity, indicating that both tyrosine and arginine residues are required for activity. A number of substitutions and modifications of the arginine residue at the carboxy terminus of the fragment were carried out. Amino acid substitutions of glutamic acid or lysine (YIGSE or YIGSK) demonstrated a significant decline in activity compared to YIGSR. The reverse sequence, RSGIY-NH_2, was significantly less active than YIGSR-NH_2 for cell adhesion. The importance of the other residues composing the pentapeptide is suggested by the observation that YGGGR lacked activity.

A quantitative dose response of HT-1080 cells to various peptides is shown in Figure 1. The level of cells attaching to 5 μg of intact laminin is shown as 100%. YIGSR-NH_2 at levels of 60 μg or above supported the attachment of a similar or even greater number of cells as did 5 μg of laminin while the other peptides (YIGSE, YGGGR, IGSR, IGSE) were inactive.

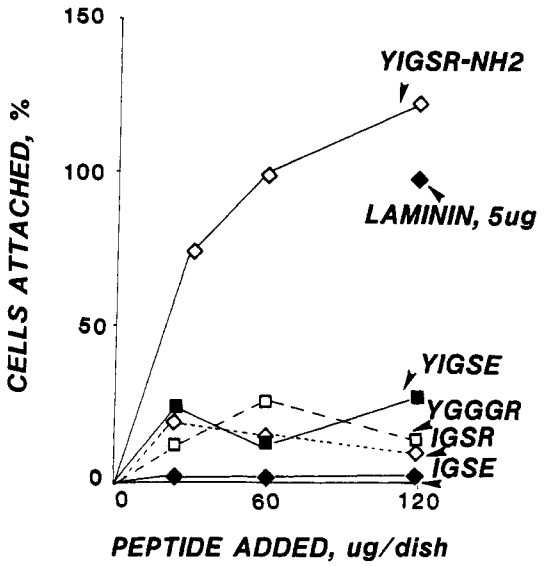

FIGURE 1: Attachment of HT-1080 cells to synthetic peptides. YIGSR-NH_2 and permutations of this active peptide were dried onto tissue culture dishes, and attachment was assayed as described under Experimental Procedures.

Table II: Ability of Various Cell Types To Attach to YIGSR-NH_2[a]

cell type	attachment (%) laminin, 5 μg/dish	attachment (%) YIGSR-NH_2, 100 μg/dish
HT-1080 (fibrosarcoma)	100	100
CHO (Chinese hamster ovary)	100	100
MCF-7 (breast carcinoma)	100	99
A431 (epidermoid carcinoma)	100	100
G-8 (muscle line)	100	98
B16F10 (melanoma)	100	85
PAM 212 (epidermal line)	100	71
NG108-15 (neuroblastoma x glioma)	100	59
chondrocytes (transformed with retrovirus)	0	0
human skin fibroblasts[b]	50	0
bovine osteoblasts[b]	20	0

[a] The attachment of these cells was tested over a range of YIGSR-NH_2 concentrations (6–250 μg/mL). Data are shown for maximal adhesion, which was observed at 60 μg/mL. Maximal attachment (80% of the added cells) to laminin was achieved with 5 μg/mL. [b] These cells attached well (~80% of the total added cells) to fibronectin.

The ability of a number of cell types to attach to YIGSR-NH_2 and to laminin (Table II) was tested. Several cell lines, including HT-1080 (fibrosarcoma), MCF-7 (breast carcinoma), A431 (epidermoid cells), and G-8 (muscle), attached as readily to YIGSR (60 μg/mL) as to laminin (5 μg/mL). Other cells, including B16F10 melanoma and PAM 212 epidermal cells, attached to a lesser extent to the peptide than to laminin, and the NG108-15 neuroblastoma x glioma cells attached well to laminin but to half the level observed with YIGSR-NH_2. Higher concentrations of peptide (up to 2.0 mg/mL) did not increase the number of cells that attached. Various other cells, including chondrocytes, fibroblasts, and osteoblasts, did not attach to YIGSR, although they showed a low but significant attachment to laminin, and the latter two had a much higher affinity for fibronectin.

A laminin receptor (M_r 67 000) has been isolated by affinity chromatography of detergent extracts of cell membranes on laminin–Sepharose (Rao et al., 1982; Malinoff & Wicha, 1983; Lesot et al., 1983). Previously, we found that the nonapeptide CDPGYIGSR eluted the laminin receptor from laminin affinity columns apparently by competing with the attachment site on laminin (Graf et al., 1987). Here we

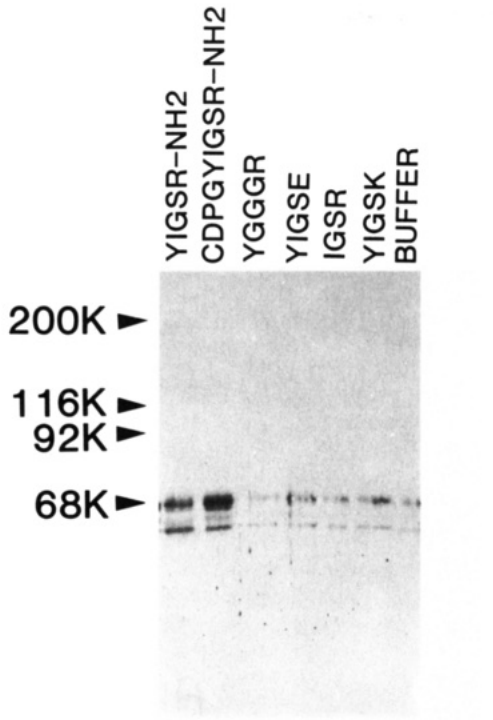

FIGURE 2: Western immunoblot with specific antibody to the laminin receptor (M_r 67 000) of the receptor eluted from laminin–Sepharose columns with synthetic peptides. Detergent extracts of EHS cell membranes were chromatographed on laminin–Sepharose columns. Nonspecifically bound material was washed from the column, and the peptides (0.6 mg/mL) were passed over parallel columns. Eluates were electrophoresed on a 7.5% SDS–polyacrylamide gel, and western immunoblot was performed by using specific antibody to the 67-kDa laminin receptor.

evaluated various peptides derived from the CDPGYIGSR sequence as well as peptides with amino acid substitutions for their ability to displace the laminin receptor from laminin–Sepharose affinity adsorbents. In these studies, an octyl glucoside extract of cell membranes isolated from EHS tumor cells was applied to laminin affinity columns, and unbound material was removed with buffers. Subsequently, various peptides were tested as eluants, and the proteins displaced by the peptide were resolved by electrophoresis, transferred to nitrocellulose paper, and reacted with antibody to the laminin receptor. Such studies showed that CDPGYIGSR eluted principally a major protein (M_r 67 000) and a minor component (M_r 63 000), which reacted with antibody to the laminin receptor prepared from EHS tumor cell membranes. ^{125}I-Labeled laminin was found to bind to both bands (data not shown). It is likely that the larger component represents the intact receptor while the smaller and less abundant component may be a degradation product. As shown in Figure 2, a low level of receptor was displaced by buffer as well as by various peptides (YGGGR, YIGSE, IGSR, YIGSK) that were not active in the cell attachment assay. In contrast, YIGSR-NH_2 eluted significantly greater amounts of receptor, and there is a reasonable correlation between receptor elution and the activity of the peptide in mediating cell attachment (Table I). As expected, a mixture of the amino acids that are present in YIGSR shows little ability to elute the receptor.

Discussion

Through the use of adhesion and receptor binding assays, we have determined that a sequence of five amino acids, YIGSR, on the B1 chain of laminin is the minimal sequence with these biological activities. The amide form of the peptide has almost twice the activity of YIGSR itself, presumably because the amide group removes the negative charge from the peptide. Chemical modification of the arginine residue with 1,2-cyclohexanedione abolished the adhesion activity of both laminin and the peptide. However, while the modification eliminated adhesive activity, the modified laminin was able to stimulate neurite outgrowth, suggesting that these are two highly specific processes controlled by different sequences in the laminin molecule (Kleinman, unpublished observation). Substitutions for arginine yielded less active peptides as did elimination of the tyrosine residue. The isoleucine and serine residues may also be necessary, since substituting glycine for these produces a less active peptide. Reversal of the sequence to RSGIY produced a less active peptide. This result is in contrast to the cell attachment site on fibronectin, RGDS, which is active in either orientation for fibronectin-mediated cell attachment (Yamada & Kennedy, 1985).

A number of cell types that attached to laminin also attached to YIGSR. It should be noted that while these cells attached well to YIGSR, no cell spreading was observed. Thus, other domains in the molecules may be involved in cell spreading. Further, YIGSR in the B1 chain may not be the sole attachment site in laminin. Neuronal cells are known to recognize the long arm of laminin (Edgar et al., 1984), and the reduced attachment of the neural hybrid line NG108-15, the B16 melanoma cells, and the PAM 212 epidermal cells to YIGSR in comparison to laminin could also be due to the affinity of the cells for other sites in laminin that would enhance their binding. A specific site on fibronectin, RGDS, has been found to be active in cell adhesion for many cell types (Pierschbacher & Ruoslahti, 1984). In addition, fibronectin has been found to have a second adhesion site, REDV, which is recognized by melanoma cells (Humphries et al., 1986). To date, REDV has not been found in the laminin molecule (Sasaki, unpublished results).

YIGSR is a unique sequence found only in the laminin B1 chain and not in either the B2 chain or the A chain (Sasaki, unpublished results). A data base search encompassing over 10 000 proteins revealed the YIGSR sequence only in one plant protein (Auperin et al., 1984). This sequence is not present in fibronectin. In contrast, RGDS, which is found in many proteins (Ruoslahti & Pierschbacher, 1986), is not present in the B1 chain of laminin. Such distinct specificity of these cell attachment sequences may help to explain the different responses these molecules elicit from different cell types (Terranova et al., 1986), which are mediated through separate cell surface receptors.

Acknowledgments

We thank Dr. Y. Iwamoto for helpful discussions and the various investigators who gave us the cells. We thank Dr. K. von der Mark for providing the laminin receptor antibody. We also thank S. Chisley for valuable assistance as well as S. Jacobs, L. Wepasnick, and R. Smith for preparation of the manuscript.

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Phosphorylation of the Multidrug Resistance Associated Glycoprotein†

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Received July 23, 1987; Revised Manuscript Received August 20, 1987

ABSTRACT: Drug-resistant cell lines derived from the mouse macrophage-like cell line J774.2 express the multidrug resistant phenotype which includes the overexpression of a membrane glycoprotein (130–140 kilodaltons). Phosphorylation of this resistant-specific glycoprotein (P-glycoprotein) in intact cells and in cell-free membrane fractions has been studied. The phosphorylated glycoprotein can be immunoprecipitated by a rabbit polyclonal antibody specific for the glycoprotein. Phosphorylation studies done with partially purified membrane fractions derived from colchicine-resistant cells indicated that (a) phosphorylation of the glycoprotein in 1 mM $MgCl_2$ was enhanced a minimum of 2-fold by 10 μM cAMP and (b) the purified catalytic subunit of the cAMP-dependent protein kinase (protein kinase A) phosphorylated partially purified glycoprotein that was not phosphorylated by [γ-^{32}P]ATP alone, suggesting that autophosphorylation was not involved. These results indicate that the glycoprotein is a phosphoprotein and that at least one of the kinases responsible for its phosphorylation is a membrane-associated protein kinase A. The state of phosphorylation of the glycoprotein, which is a major component of the multidrug resistance phenotype, may be related to the role of the glycoprotein in maintaining drug resistance.

Selection of cultured mammalian tumor cells for resistance to the cytotoxic effects of natural products often results in the expression of a complex set of characteristics referred to as the multidrug resistant (mdr) phenotype. The characteristics of this phenotype include (a) cross resistance to functionally and structurally unrelated drugs, (b) a net decrease in drug accumulation, (c) overexpression of a multidrug resistance associated plasma membrane glycoprotein (MDRG;[1] also known as P-glycoprotein), and (d) amplification and expression of the gene(s) coding for the MDRG. The importance of the MDRG in maintaining the mdr phenotype is exemplified by resistant cells that revert to drug sensitivity in the absence of drug and no longer overexpress the MDRG (Roy & Horwitz, 1985). Recent studies suggest that the MDRG may function

† Supported in part by USPHS Grants CA-39821 and GM-34555 and American Cancer Society Grant CH-86.
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[1] Abbreviations: MDRG, multidrug resistance associated glycoprotein; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; WGA, wheat germ agglutinin; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/87/0426-6900$01.50/0