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The 90-Kilodalton Peptide of the Heme-Regulated eIF-2α Kinase Has Sequence Similarity with the 90-Kilodalton Heat Shock Protein†

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ABSTRACT: Highly purified preparations of the heme-controlled eIF-2α (eukaryotic peptide initiation factor 2 α subunit) kinase of rabbit reticulocytes contain an abundant 90-kilodalton (kDa) peptide that is immunologically cross-reactive with spectrin and that modulates the activity of the enzyme [Kudlicki, W., Fullilove, S., Read, R., Kramer, G., & Hardesty, B. (1987) *J. Biol. Chem. 262*, 9695–9701]. The amino-terminal sequence of the 90-kDa protein has a high degree of similarity with the known amino-terminal sequences of the *Drosophila* 83-kDa heat shock protein (20 out of 22 residues) and with other related heat shock proteins. The amino acid sequence of a tryptic phosphopeptide isolated by high-performance liquid chromatography from the eIF-2α kinase associated 90-kDa protein after phosphorylation by casein kinase II is shown to be identical with a 14 amino acid segment of the known sequence of the *Drosophila* 83-kDa heat shock protein. Results of hydrodynamic studies indicate a highly elongated structure for the reticulocyte protein, characteristic of a structural protein. Additional structural similarities between the eukaryotic heat shock proteins, the reticulocyte eIF-2α kinase associated 90-kDa peptide, and spectrin are discussed.

Protein synthesis in rabbit reticulocytes is controlled by the availability of heme. In the absence of heme, a translational inhibitor, called the heme-controlled repressor (HCR)[1] (Gross & Rabinovitz, 1972), is activated in reticulocytes or their cell-free lysates. An enzyme system consisting of several constituent peptides has been shown to be responsible for the observed inhibition and possesses protein kinase activity toward the smallest or α subunit of eukaryotic peptide initiation factor 2, eIF-2 [reviewed in Ochoa (1983) and Hardesty et al. (1985)]. Highly purified preparations of the heme-controlled kinase contain a relatively abundant 90-kDa peptide as judged by SDS/PAGE. This peptide does not phosphorylate eIF-2α and does not inhibit the eIF-2-mediated binding of Met-tRNA$_f$ to 40S ribosomal subunits (Wallis et al., 1980). Rather, it appears to effectively increase the activity of the kinase that mediates these effects (Kudlicki et al., 1987).

Recently, a similarity between the 90-kDa peptide associated with eIF-2α kinase and the β subunit of spectrin, the most abundant component of the erythroid membrane skeleton (Bennett, 1985), was demonstrated (Kudlicki et al., 1985). The evidence for relatedness of the two proteins involves immunological cross-reactivity of antibodies from several distinct monoclonal hybridomas derived from different fusions and apparent similarities as substrates for two different protein kinases. Spectrin α or β subunits as well as the authentic 90-kDa peptide increase the enzymatic activity of the eIF-2α kinase, and some of the antibodies raised against spectrin affect the biological activity of the kinase. The association of the eIF-2α kinase with spectrin-related peptides has led to the development of a model in which this enzyme system essential for the modulation of protein synthesis interacts with (and possibly is regulated by) structural components of the cell (Hardesty et al., 1985).

Heme deprivation appears not to be the only stimulus responsible for the activation of HCR. It has been shown in intact reticulocytes as well as their cell-free lysates (Ernst et al., 1982), and more recently in HeLa cells (Duncan & Hershey, 1984; de Benedetti & Baglioni, 1986), that the inhibition of protein synthesis associated with the heat shock response correlates with the phosphorylation of eIF-2α. Furthermore, the kinase responsible for this phosphorylation appears to be sensitive to heme and in one case (de Benedetti & Baglioni, 1986) was shown to be inhibited by polyclonal anti-HCR antibodies. It seems likely that eIF-2α phosphorylation by an enzyme analogous to HCR has at least a contributory effect on translational inhibition subsequent to heat shock (Burdon, 1986).

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[1] Abbreviations: HCR, heme-controlled repressor; NBRF, National Biomedical Research Foundation; HPLC, high-performance liquid chromatography; TSTA, tumor-specific transplantation antigen; eIF-2, eukaryotic peptide initiation factor 2; eIF-2α, smallest subunit of eIF-2; SDS/PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; RP, reversed phase; kDa, kilodalton(s); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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In this paper we present evidence that the 90-kDa peptide associated with the reticulocyte heme-controlled eIF-2α kinase has extensive similarity with the 83–90-kDa protein synthesized by eukaryotic cells in response to heat shock and probably is physically and functionally identical. Apparent structural similarities between the 90-kDa reticulocyte protein, spectrin, and eukaryotic 83–90-kDa heat shock proteins are discussed.

Experimental Procedures

Materials. [γ-^{32}P]ATP was purchased from New England Nuclear. Phosvitin and CNBr-activated Sepharose were from Sigma; other chromatography media and chemicals were used as previously indicated (Kudlicki et al., 1985, 1987). Computer analysis of sequences and homology searches were performed by using the Beckman Microgenie library of routines.

Isolation of Proteins from Reticulocytes. Fractionation of the rabbit reticulocyte postribosomal supernatant to give the PC_{100} fraction has been described (Wollny et al., 1984). The eIF-2α kinase was further purified and the 90-kDa protein isolated as reported (Kudlicki et al., 1985). Casein kinase II was purified from a protein fraction that adhered to phosphocellulose [cf. Figure 1 in Wollny et al. (1984)] and was eluted between 0.4 and 0.8 M KCl. This fraction was diluted with 20 mM Tris-HCl (pH 7.5) to give 0.25 M KCl. It was then loaded on a phosvitin–Sepharose column, prepared according to instructions provided by Pharmacia, and equilibrated in 20 mM Tris-HCl (pH 7.5) and 0.25 M KCl. The matrix was washed; then protein was batch-eluted by increasing the salt concentration first to 0.35 M and then to 0.45 M. The enzyme was obtained in the latter fraction. Incorporation of [^{32}P]phosphate into protein substrates was analyzed by SDS/PAGE (Grankowski et al., 1979) followed by autoradiography under X-Omat film (Kodak).

Phosphorylation and Isolation of the Phosphorylated 90-kDa Peptide. Phosphorylation of the reticulocyte 90-kDa peptide (about 100 μg) was carried out in a total volume of 400 μL containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2.5 mM dithioerythritol, 2 μg of casein kinase II, and 0.12 mM [γ-^{32}P]ATP (2–3 Ci/mmol). Incubation was for 20 min at 35 °C. The 90-kDa phosphopeptide was reisolated by HPLC using a SynChropak GPC 300 column in a Beckman/Altex system as described previously (Wollny et al., 1984).

Glycerol Gradient Centrifugation and Molecular Sieve Chromatography. These procedures were carried out with about 15 μg of ^{32}P-labeled 90-kDa protein under conditions identical with those reported previously for analysis of the eIF-2α kinase (Kudlicki et al., 1987).

Analysis of Tryptic Peptides Derived from the 90-kDa Phosphopeptide. The phosphorylated 90-kDa peptide (about 80 μg) was digested with trypsin (1.6 μg) in 500 μL of 100 mM NH_4HCO_3 (pH 7.8) for 1 h at 37 °C. An equal volume of 6 M guanidine hydrochloride containing 2 mM EDTA and 2% trifluoroacetic acid (TFA) was added. The sample was clarified by centrifugation, and the tryptic peptides were analyzed by RP-HPLC using a Bakerbond C_8 column fitted with a Brownlee RP300 C_8 guard column equilibrated in 5% acetonitrile in aqueous 0.1% TFA. Peptides were eluted with a gradient from 0 to 50% acetonitrile over 90 min. The flow rate was 1 mL/min. ^{32}P radioactivity of the eluted fractions was determined by Cerenkov counting.

General Sequencing Procedures. Automated Edman degradations were performed on an Applied Biosystems gas/liquid-phase Model 470A sequencer (Hewick et al., 1981), and the phenylthiohydantoin derivatives were analyzed by RP-HPLC using a Zorbax ODS column (Zimmerman et al., 1977). Samples were dissolved in 50% (v/v) trifluoroacetic acid for application to the polybrened disk; 100% trifluoroacetic acid rinses of the sample tubes were also applied to the disk. Standard sequencing conditions using trifluoroacetic acid conversion chemistry were employed; the acetonitrile used for extraction of phenylthiohydantoin residues from the flask was supplemented with dithiothreitol (10 mg/200 mL).

Phosphoamino Acid Analysis. Acid hydrolysis and thin-layer electrophoresis of phosphopeptides isolated by RP-HPLC were carried out as described in Hunter and Sefton (1980) with minor modifications. Silica gel plates from Eastman Kodak were used and electrophoresis at pH 1.9 was run for 4 h at 400 V.

Results

Hydrodynamic Properties of the 90-kDa eIF-2α Kinase Associated Peptide. The sedimentation constant ($s_{20,w}$ = 6.0) and the apparent Stokes radius (83 Å) of the 90-kDa protein were determined by comparison of the highly purified protein with well-characterized standard proteins. Glycerol density gradient centrifugation was used for the sedimentation constant, and molecular sieve chromatography was used for the Stokes radius. The position of the ^{32}P-labeled protein was identified by scintillation counting in both cases. The molecular weight of the protein was calculated from the experimentally determined values for the Stokes radius and sedimentation coefficient and found to be 2.2×10^5, assuming that the protein had a partial specific volume of 0.74 cm^3/g. This corresponds to a frictional coefficient, f, of 1.6×10^{-7} g s^{-1}, whereas a typical spherical protein would be expected to have a frictional coefficient, f_0, of 7.8×10^{-8} g s^{-1}. Thus, f/f_0 for the protein is estimated to be 2.05. For a protein of typical density and of the form of a prolate ellipsoid this corresponds to an axial ratio of 21:1 (Tanford et al., 1961). The uncertainty involved in these types of measurements notwithstanding, the results indicate a highly asymmetric solution structure characteristic of structural proteins rather than globular enzymes. This appears to be an additional characteristic of the 90-kDa protein shared by spectrin, which is known to be highly elongated (Calvert et al., 1980).

Amino-Terminal Sequence Analysis of the Reticulocyte 90-kDa Protein. To further characterize the eIF-2α kinase associated 90-kDa peptide, automated sequencing was performed on the purified protein. An unambiguous sequence was obtained for the first 32 amino acid residues beginning with an amino-terminal proline residue as presented in Figure 1. This sequence was used for a computer-assisted homology search with other known proteins in the NBRF data base. It was found that this sequence was highly similar to the amino-terminal region of the *Drosophila* 83-kDa heat shock protein (Lis & Hackett, 1983) and to the analogous 90-kDa protein from yeast (Farelly & Finkelstein, 1984) (Figure 1). The extent to which this sequence can be aligned with other known heat shock protein sequences leaves little question that the reticulocyte protein is closely related. An amino-terminal extension of 10–13 amino acids is present in the rabbit protein, followed by very close agreement with both the *Drosophila* and yeast sequences. Chicken hsp108, a protein induced in chicken oviduct cells by heat shock (Sargan et al., 1986), also possesses an amino-terminal extension, in this case of over 60 amino acids, that precedes the aligned segments of the *Drosophila* and yeast sequences. In addition, when the sequence is compared to the amino-terminal sequence of the murine 86-kDa tumor-specific transplantation antigen (TSTA) from the methylcholanthrene-induced tumor meth A (Ullrich et al., 1986), it is found that 25 of a possible 30 amino acids are

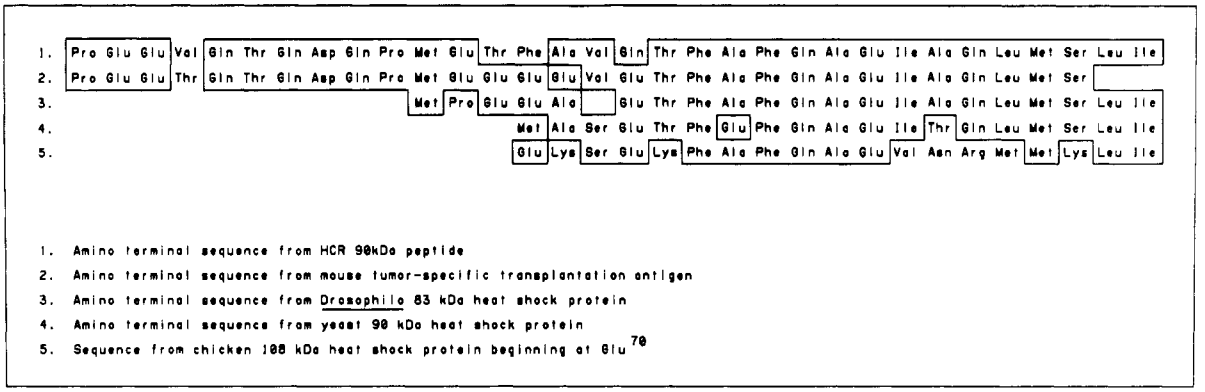

FIGURE 1: Amino-terminal sequence of the 90-kDa eIF-2α kinase associated protein and its similarity to eukaryotic heat shock proteins. Comparison of sequences obtained by protein sequencing methods (1 and 2) and by deduction from nucleotide sequences (3–5) is shown. Regions of similarity are boxed.

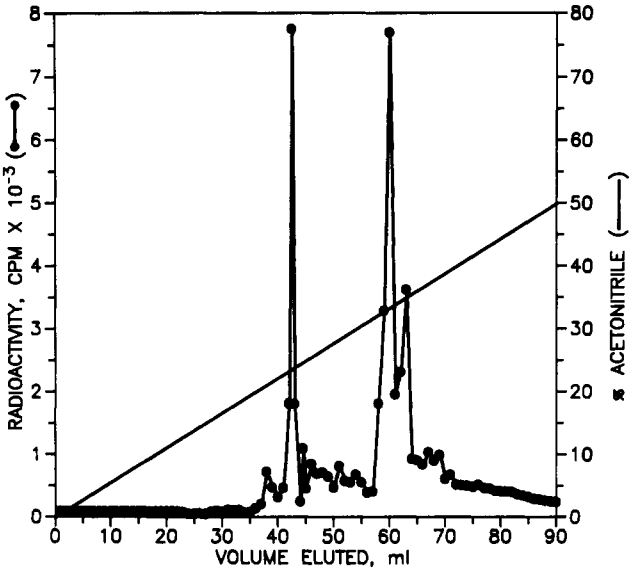

FIGURE 2: Isolation of tryptic phosphopeptides derived from the HCR kinase associated 90-kDa protein. Phosphorylation of the 90-kDa protein by casein kinase II was carried out with [γ-^{32}P]ATP as described under Experimental Procedures. About 80 μg of the phosphorylated protein was incubated with 1.6 μg of trypsin at 37 °C for 1 h. The resulting peptides were separated by HPLC using a reversed-phase C_8 column. Radioactivity of the resulting fractions was determined by Cerenkov counting.

identical. The TSTA sequence also contains an amino-terminal extension of 10 amino acids of which 9 are identical with the kinase-associated protein. This TSTA has been shown to be a heat shock related protein (Ullrich et al., 1986).

Amino Acid Sequence of a Phosphopeptide Derived from the Reticulocyte 90-kDa Peptide. The reticulocyte 90-kDa protein was phosphorylated by casein kinase II by using [γ-^{32}P]ATP as described under Experimental Procedures. Trypsin digestion of the phosphoprotein was then carried out, followed by analysis of the digested fraction by HPLC using a C_8 reversed-phase column as described under Experimental Procedures. The elution profile of phosphopeptides from the trypsin digest is shown in Figure 2. A major phosphopeptide eluting at 22.5% acetonitrile, thus termed T-22, was detected. The phosphopeptide was repurified by HPLC using a shallower acetonitrile gradient, and amino acid sequence analysis of the phosphopeptide was carried out by automated Edman degradation as described under Experimental Procedures. An unambiguous sequence shown in Figure 3 was obtained for T-22. This sequence was compared to known heat shock protein sequences and to protein sequences stored in the NBRF data base to find potential casein kinase II phosphorylation

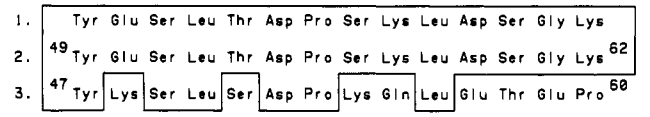

FIGURE 3: Sequence of phosphopeptide eluting from RP-HPLC at 22% acetonitrile (cf. Figure 2) and its similarity to heat shock proteins. The complete sequence of a tryptic phosphopeptide derived from the reticulocyte eIF-2α kinase associated 90-kDa protein is shown in (1). Similarity to 83–90-kDa heat shock proteins is indicated by comparison to known sequences from (2) *Drosophila* (Lis & Hackett, 1983) and (3) yeast (Farelly & Finkelstein, 1984).

sites in other protein substrates. A perfect match was found with only one sequence, which corresponded to amino acids 49–62 of the *Drosophila* 83-kDa heat shock protein (Lis & Hackett, 1983). Strong similarity also was found with the sequence of the yeast 90-kDa heat shock protein (Farelly & Finkelstein, 1984) between residues 47 and 60, as shown in Figure 3.

Phosphoamino Acid Analysis of the Isolated Phosphopeptide. The T-22 phosphopeptide contains three serine residues and one threonine residue, each of which represents a potential phosphorylation site for casein kinase II. The T-22 phosphopeptide was further analyzed to determine which amino acid residues within the sequence might be covalently modified. After acid hydrolysis of the phosphopeptide followed by thin-layer electrophoresis at pH 1.9 (Hunter & Sefton, 1980), radioactivity was found to be associated with both phosphoserine and phosphothreonine to about the same extent (data not presented). Thus, phosphorylation by casein kinase II apparently occurs at least at one of the three serine residues in the phosphopeptide and also at the threonine residue corresponding to Thr-53 of the *Drosophila* sequence. It is not possible to state from these data, however, that casein kinase II phosphorylates one serine and the threonine site at the same rate, because the phosphorylation state of the 90-kDa protein as it is isolated from reticulocytes has not been determined. Attempts to fully phosphorylate the 90-kDa protein by casein kinase II in vitro have resulted in incorporation of approximately 0.7 mol of phosphate/mol of protein. This observation, in light of the presence of multiple phosphorylation sites in the protein by this kinase, seems to indicate that it may exist largely in the phosphorylated state as has been shown for spectrin (Usui et al., 1983).

Discussion

The extent of and functional basis for the structural similarities between the 90-kDa eIF-2α kinase associated peptide, spectrin, and the 83–90-kDa eukaryotic heat shock proteins are not fully understood. A survey of the published charac-

teristics of these proteins reveals a number of distinct similarities in addition to those reported here and briefly described in the introduction. Each has an apparently elongated structure as judged by hydrodynamic studies and other methods. Sedimentation constant and Stokes' radius values similar to those reported here have been obtained for the 90-kDa heat shock protein of HeLa cells (Welch & Feramisco, 1982). Recently, the 90-kDa heat shock protein of mouse lymphoma cells was shown to exist as a homodimer under physiological conditions and to bind actin filaments (Koyasu et al., 1986). This binding is regulated by calmodulin in a Ca^{2+}-dependent manner (Nishida et al., 1986). Spectrin cross-links actin filaments (Cohen et al., 1980), and calmodulin–Sepharose chromatography has been used as an affinity purification method for the isolation of β-spectrin (Sears et al., 1986). The published partial amino acid sequences for spectrin and the 83–90-kDa heat shock proteins reveal that both contain acidic regions with many glutamic and aspartic acid residues. Direct comparison of heat shock protein sequences with the known sequences from both subunits of spectrin (Speicher & Marchesi, 1984) does not reveal extensive similarity; however, only partial spectrin sequences are currently available. The heat shock proteins of yeast and *Drosophila* do not appear to possess the 106 amino acid repetitive primary structure shown to exist in spectrin (Speicher & Marchesi, 1984; Birkenmeier et al., 1985). The sequence Glu-Asp-Leu-Thr-Asp-Pro, similar to part of the T-22 phosphopeptide sequence shown in Figure 3, is present in chicken nonerythroid α-spectrin near its C-terminus in a region of low similarity to the rest of the molecule (Birkenmeier et al., 1985). The carboxy-terminal region of β-spectrin, which has been shown to contain all of the phosphorylation sites (Harris & Lux, 1980), is known to be structurally dissimilar to the remainder of the molecule and has not been sequenced to date. The immunological cross-reactivity of the 90-kDa HCR-associated protein and spectrin from reticulocytes has been demonstrated by using monoclonal antibodies (Kudlicki et al., 1987). Extracts obtained from HeLa cells after hyperthermia also contain an immunoreactive peptide of 90 kDa and several other higher molecular weight peptides possibly derived from spectrin which react with these antibodies.[2] Further structural characterization of spectrin and the 90-kDa peptides is required to demonstrate differences and similarities in their physical structure.

We find the apparent association between eIF-2α kinase and a eukaryotic heat shock protein to be intriguing in its physiological implications. Kinase activity directed toward the α subunit of eIF-2 is markedly enhanced by the 90-kDa spectrin-related peptide as well as by the intact α or β subunits of spectrin itself (Kudlicki et al., 1987). Association of other protein kinases with 83–90-kDa heat shock proteins has previously been observed in the Rous sarcoma virus src oncogene product (Oppermann et al., 1981), in several other tyrosine kinases (Ziemecki et al., 1986), and most recently with casein kinase II itself (Dougherty et al., 1987).

Induction of synthesis of the 90-kDa heat shock protein by hyperthermia is associated with inhibition of normal cellular translation. The mechanism of this inhibition is the subject of some debate as several distinct modifications to the translational apparatus have been suggested as the underlying cause (Panniers et al., 1985; Kennedy et al., 1984). It has been shown, however, that the phosphorylation of eIF-2α in response to heat shock does occur in HeLa cells (Duncan & Hershey, 1984) and that the responsible kinase is sensitive to heme and inhibited by anti-HCR antibodies (de Benedetti & Baglioni, 1986). It seems likely that activation of eIF-2α kinase during heat shock has at least a contributory effect upon translational inhibition. It is therefore of interest that one of the proteins specifically induced during the heat shock process may have the potential to modulate the activity of the eIF-2α kinase.

[2] Unpublished results.

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Rapid Increases in the Transglutaminase Activity of A431 Cells following Treatment with Epidermal Growth Factor

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ABSTRACT: Transglutaminase activity was detected in lysates of A431 cells, a human epidermal carcinoma cell line. Enzyme activity was increased 1.5–2.5-fold in lysates prepared from cells pretreated with epidermal growth factor (EGF) relative to untreated control cells. Half-maximal activation of the transglutaminase activity occurred at 3–5 nM EGF, a concentration in good agreement with the K_d for EGF binding to its receptor in these cells. The increase in transglutaminase activity could be detected as early as 2 min after the addition of EGF, with the maximal response attained by 30 min. The activation was not blocked by pretreatment of the cells with cycloheximide, suggesting that the increased activity was not the result of an induction of transglutaminase synthesis. Fractionation of A431 cell lysates by centrifugation at 100000*g* for 30 min demonstrated that 90% of the transglutaminase activity was present in the soluble fraction and that this soluble transglutaminase activity was increased after treatment of the cells with EGF. The demonstration that EGF acutely increases the activity of a soluble, intracellular transglutaminase defines a novel pathway of growth factor action and provides a useful model system for identifying and comparing the mechanism(s) by which growth factors activate soluble enzymes.

Epidermal growth factor (EGF)[1] is a low molecular weight polypeptide that binds to a specific cell surface receptor and stimulates the phosphorylation of proteins on tyrosine residues (Carpenter & Cohen, 1979; Carpenter et al., 1978, 1979). While it seems likely that the intracellular effects of EGF are mediated via this increase in protein phosphorylation, little data are available to support this hypothesis. To develop a system in which to study the mechanism of signal transduction by EGF, we sought to identify an intracellular enzyme, the activity of which was regulated by EGF.

Transglutaminases are a family of Ca^{2+}-dependent enzymes that catalyze the formation of ϵ-(γ-glutamyl)lysine–protein cross-links and the incorporation of primary amines into protein-bound glutamine residues (Folk, 1980). These enzymes have been found in a wide range of species from sea urchins to mammals and in many different cell types (Folk, 1980; Cariello et al., 1984). Transglutaminase activity has been detected in both intracellular and extracellular locations. Formation of ϵ-(γ-glutamyl)lysine cross-links generally leads to a stabilization of protein structure. For example, factor XIIIa is a plasma transglutaminase responsible for the stabilization of fibrin clots. Several specific intracellular transglutaminases have been shown to be involved in the formation of cornified envelopes (Thacher & Rice, 1985) and the cross-linking of hair fiber protein (Chung & Folk, 1972; Harding & Rogers, 1972). While the biological role of a few specific transglutaminases has been defined, the function of the majority of intracellular transglutaminases remains unclear. Intracellular transglutaminases have been postulated to be involved in cell adhesion (Slife et al., 1986), cytoskeletal stabilization (Maccioni & Arechaga, 1986), cell growth and differentiation (Birckbichler & Patterson, 1978), and receptor-mediated endocytosis (Davies et al., 1980; Fesus et al., 1984).

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[1] Abbreviations: EGF, epidermal growth factor; HBSS, Hank's balanced salts solution; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; PI, phosphatidylinositol.

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