Ubiquitination of Na,K-ATPase α1 and α2 subunits

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Received 22 January 1997

Abstract Na,K-ATPase, an essential transporter of mammalian cells, is an oligomeric transmembrane protein composed of two subunits, α and β, of which there are several isoforms. In this study, we demonstrate that the α1 and α2 isoforms of the Na,K-ATPase α subunit are modified by the covalent attachment of ubiquitin polymers in COS-7 cells. We propose that polyubiquitination of the Na,K-ATPase α subunit may play a role in regulating its degradation.

Key words: Na,K-ATPase; Ubiquitin; Polytopic integral membrane protein; COS-7 cell; α subunit; Proteasome

1. Introduction

Na,K-ATPase both establishes and maintains the electrochemical gradient across the plasma membrane of mammalian cells by coupling the hydrolysis of ATP to the transport of sodium and potassium ions [1,2]. It is an oligomeric membrane protein composed of two subunits, α and β, of which there are several isoforms [1,2]. The subunit composition of the Na,K-ATPase affects its sensitivity to various hormones [3] as well as its affinity for cations [4–9] and ouabain, a specific inhibitor of the Na,K-ATPase [2]. Both the α and β subunits of Na,K-ATPase are integral membrane proteins which are cotranslationally inserted into the membrane of the endoplasmic reticulum. Association of the two subunits and maturation of Na,K-ATPase into a functional transporter also occur in this compartment [10–12]. Properly folded α/β heterodimers enter the secretory pathway and are eventually transported to the cell surface, whereas unassembled subunits are retained in the endoplasmic reticulum and degraded [10,12]. In *Xenopus* oocytes the half-life of the unassociated α subunit ($t_{1/2} \approx 2$ h) is 10-fold lower than that of the mature α/β heterodimer [12]. Little, however, is known about the mechanism of the degradation of Na,K-ATPase and its individual subunits.

The intracellular degradation of proteins is thought to be due primarily to the combined action of the proteasome, a multi-subunit cytosolic protease of broad specificity, and the lysosome, an acidic compartment of the cell in which numerous proteases and glycosidases are located. The covalent attachment of polyubiquitin chains to proteins frequently serves as a signal for their degradation by the proteasome [13]. Originally, the proteasome was thought to be involved primarily in the degradation of soluble cytosolic proteins [13]. Recently, however, the proteasome has been implicated in the degradation of misfolded proteins which are retained in the endoplasmic reticulum [14]. Polyubiquitination of proteins may also be involved in the internalization and degradation of plasma membrane proteins, a process which may entail the combined action of the proteasome and the lysosome [13,15].

Here we demonstrate polyubiquitination of both the α1 and α2 isoforms of the Na,K-ATPase α subunit in COS-7 cells. The Na,K-ATPase α subunit may therefore be a member of the growing list of integral membrane proteins that are degraded, at least partially, by the proteasome.

2. Materials and methods

2.1. Materials

All reagents used to culture cells were purchased from Gibco BRL (Grand Island, NY). The α1 specific polyclonal rabbit antibody 620 was raised against rat kidney Na,K-ATPase by Dr. Jonathan Lytton in this laboratory. The α2 isoform specific polyclonal rabbit antibody 164 was raised against a peptide corresponding to amino acids 459–472 of the α2 subunit of Na,K-ATPase by Diana McGill in this laboratory. The 12CA5 monoclonal antibody which recognizes the influenza virus hemagglutinin epitope was obtained from the Monoclonal Antibody Facility of Harvard University (Cambridge, MA). Dr. P. Martin-Vasallo (University of Tenerife, Tenerife) kindly provided us with the β1 specific polyclonal antibody, SPETβ1 [16]. Finally, Dr. A.L. Haas (Medical College of Wisconsin, Milwaukee, WI) generously provided us with a sample of the affinity-purified rabbit polyclonal antibody against SDS purified ubiquitin [17].

2.2. Molecular biology

The wild type α2 coding sequence was excised from a variant of the vector rα2-pKC-4 [4] (obtained from Dr. Jerry Lingrel, University of Cincinnati, Cincinnati, OH), blunted, ligated to *Pst*I linkers, and inserted into the *Pst*I site of the mammalian expression vector pMT2 [18] (obtained with permission from the Genetics Institute, Cambridge, MA). The substitutions L111R and N122D were introduced into a pGem3zf- vector containing the first 673 base pairs of the α2 coding region by the method of Kunkel et al. [19]. The vector rα1-pKC-4 [20] was also obtained from Dr. Jerry Lingrel. The vector pCDNA1 FNα1 was constructed as previously described [21]. Dr. Kyung Lim Yoon in our laboratory used a similar strategy to construct pCDNAI FNα2S364P, a vector directing the expression of a mutant of the α2 subunit, containing a substitution at serine 364, with the influenza virus hemagglutinin epitope fused to its N-terminus. To generate an epitope tagged wild type α2 subunit, a restriction fragment containing the epitope tag and the first seven residues of the α2S364 coding sequence was used to replace the corresponding fragment of the wild type α2 subunit cDNA. The resulting epitope tagged α2 subunit (FNα2) was subsequently inserted into the mammalian expression vector pCDNA I (Invitrogen, San Diego, CA). The coding sequence of the N-terminus of FNα2 is:

[*Influenza* virus hemagglutinin epitope] [α2 N-terminus.....
ATG-GTA-[TAC-CCA TAT GAC-GTC-CCG-GAC-TAC-GCC]-[GGA-CGT-GGG-GCA-GGG-CGT-
met-val [tyr-pro-tyr-asp-val-pro-asp-tyr-ala]-[gly-arg-glu ala-gly-arg-

2.3. Cell culture and transfection

COS-7 cells were grown in a humidified incubator with 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium supplemented with penicillin, glutamine, and 10% fetal bovine serum. 100 mm plates of COS-7 cells were transfected with 5 μg of supercoiled plasmid DNA by the DEAE dextran method [22] or with the lipofectamine reagent (Gibco BRL) according to the manufacturer's instructions.

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Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator

PII S0014-5793(97)00182-8

2.4. Preparation of crude membranes

Four 100 mm plates of transfected COS-7 cells were washed twice with 10 ml of phosphate buffered saline (PBS) containing 136.9 mM NaCl, 2.7 mM KCl, 8.0 mM Na_2HPO_4, and 1.5 mM KH_2PO_4. Following a 5 min incubation in PBS supplemented with 5 mM Na_2EDTA, cells were scraped off the plate with a rubber policeman, pelleted for 3 min at room temperature at $1000\times g_{av}$, and resuspended in 2 ml of ice-cold hypotonic lysis buffer containing: 50 mM sucrose, 10 mM HEPES pH 7.4, 2.5 μg/ml aprotinin, and 1 μg/ml of leupeptin, chymostatin, and pepstatin. The cells were homogenized by 25 strokes of a Dounce homogenizer with a tight pestle. Following the addition of 246 μl of 65% sucrose (w/w) in 10 mM HEPES pH 7.4 and 4 μl 0.5 M $MgCl_2$, the homogenate was subjected to two 10 min spins at $1000\times g_{av}$ at 4°C to pellet nuclei, mitochondria, and unlysed cells. Crude membranes were pelleted from the resulting supernatant by centrifugation at $100\,000\times g_{av}$ for 30 min at 4°C, washed quickly in 2 ml of hypotonic lysis buffer and resuspended in the same buffer. Protein concentration was determined by Peterson's modification of the Lowry assay [23].

2.5. Fractionation of crude membranes

The cell fractionation procedure was a modification of the method of Saraste et al. [24]. The crude membrane pellet from four 100 mm plates of COS-7 cells was resuspended in 0.7 ml of 10 mM HEPES pH 7.4 with 15 strokes of a Thomas 0448 teflon pestle homogenizer, combined with 2.3 ml of 65% (w/w) sucrose in 10 mM HEPES pH 7.4 to give a final sucrose concentration of 52.5%, and placed at the bottom of an SW41 centrifuge tube. The sample was subsequently overlaid with 1 ml of the following sucrose solutions: 45%, 40%, 35%, 30%, 25%, 20%, 15%, and 10% (w/w in 10 mM HEPES pH 7.4). Following centrifugation at 4°C for 18 h at $84\,000\times g_{av}$, 0.75 ml fractions were collected from the bottom of the tube, diluted in 6 ml of ice cold PBS containing 16.5 μg/ml aprotinin, and spun for 1 h at $100\,000\times g_{av}$ at 4°C to pellet the membranes. The membrane pellets were solubilized directly in 100 μl of reducing sample buffer.

2.6. Electrophoresis and immunoblotting

Samples were solubilized in reducing sample buffer (2% SDS, 10% glycerol, 1% β-mercaptoethanol, and 65 mM Tris-HCl pH 6.8) and heated for 5–10 min at 65°C prior to loading on SDS polyacrylamide gels. Deglycosylation of crude membranes was required to detect the COS-7 β1 subunit with the polyclonal antibody SPETβ1 [16]. Crude membranes (25 μg) were mixed with 5% SDS to give a final concentration of 0.1% SDS in 12 μl and denatured by incubating for 3 min at 65°C. Samples were deglycosylated for 18 h at 37°C in a mixture containing: 0.4 units of glycopeptidase F (Calbiochem, La Jolla, CA), 15 mM Na_2EDTA, 0.75% Nonidet P-40, 20 mM Na_2HPO_4 pH 7.2, and 10 mM β-mercaptoethanol. Deglycosylation was terminated by the addition of reducing sample buffer. Unless otherwise indicated, proteins were transferred to nitrocellulose by the procedure of Towbin [25]. To detect ubiquitination, proteins were transferred according to Haas [26] for 18 h at 100 mAmp in a buffer containing 0.01% SDS, 20 mM Tris, and 144 mM glycine. Immunoreactive bands were visualized with the Renaissance chemiluminescence reagent (Dupont), and immunoblots were stripped according to the enclosed specifications.

2.7. Immunoprecipitation

Epitope tagged α subunits were immunoprecipitated from 1 ml of lysate with 2 μg of 12CA5 monoclonal antibody and ~50 μl of fresh protein A sepharose according to Tamkun and Fambrough [27] with the following modifications. The wash with buffer containing 0.1% SDS was omitted. Three days post transfection, lysates were prepared from 100 mm plates of COS-7 cells. Plates were washed twice in 10 ml PBS and the cells solubilized by a 1 h 4°C incubation with gentle rocking in 2 ml of extraction buffer containing: 0.5% Triton X-100, 2 mg/ml bovine serum albumin, 2 mM *N*-ethyl-maleimide, 1 mM phenylmethylsulfonyl fluoride, 2.5 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml chymostatin, 1 mM EDTA, 150 mM NaCl, and 50 mM Tris-HCl pH 7.4. Bound proteins were eluted in reducing sample buffer supplemented with 1 mM dithiothreitol for 20 min at 37°C. Prior to loading, samples were heated for 5 min at 65°C.

3. Results and discussion

The endogenous Na,K-ATPase of COS-7 cells is composed of α1 (Fig. 1A, lane 1) and β1 subunits (Fig. 1B, lane 1). Transfection of COS-7 cells with a vector directing expression of the rat α1 subunit (lane 2 of Fig. 1A), results in a dramatic increase in the amount of α1 immunostaining as compared to that present in untransfected cells (lane 1 of Fig. 1A) or cells expressing the rat α2 subunit (lane 3 of Fig. 1A). In contrast, as shown in Fig. 1B, the amount of β1 subunit immunostaining is very similar in the three membrane preparations. The high levels of expression typically achieved in COS-7 cells [28], coupled with our failure to detect an increase in β1 subunit expression, suggest that the exogenous rat α subunits consist of a mixture of unassembled α subunits and α subunits that have been incorporated into functional Na,K-ATPase molecules.

Ubiquitin is a 76 amino acid (~8.5 kDa) polypeptide that is covalently linked to proteins through the formation of an isopeptide bond between its carboxyl terminus and the ε-amino group of lysine residues [13]. The covalent attachment of ubiquitin monomers and polyubiquitin chains of various lengths to the cytosolic lysine residues of a polypeptide typically results in the appearance of a smear or ladder of higher molecular weight forms. Because polyubiquitination of proteins serves as a signal for their degradation by the proteasome, a smear of partially degraded lower molecular weight forms is often visible as well [13,26]. We began to suspect that the α1 subunit was polyubiquitinated when we overexposed the immunoblot shown in Fig. 1A. As shown in lane 2 of Fig. 1C, the α1 immunostaining in COS-7 cells expressing rat α1 is clearly consistent with polyubiquitination of the protein. It is strikingly diffuse and comprises a mixture both higher and lower molecular weight forms of the protein.

As shown in lane 3 of Fig. 1D, the electrophoretic mobility of the rat α2 subunit in crude COS-7 membranes does not appear to be heterogeneous. However, when crude membranes are further purified by fractionation over a discontinuous sucrose gradient (Fig. 1E), a smear of higher molecular weight forms of the rat α2 subunit becomes apparent. This suggests that the α2 subunit is also polyubiquitinated when it is expressed in COS-7 cells. Our difficulty in detecting polyubiquitinated forms of the α2 subunit may be due to the fact that our α2 specific antibody, antibody 164, is considerably weaker than our α1 specific antibody. Another possibility is that polyubiquitination interferes with the recognition of the α2 subunit by antibody 164. Polyubiquitination can occur at multiple cytosolic lysine residues [15,29]. Antibody 164 was raised against a lysine containing peptide in the large cytosolic loop of the α2 subunit.

Because Na,K-ATPase frequently aggregates and migrates aberrantly in the presence of detergents and reducing agents, we were concerned that the high molecular weight forms of the α2 and α1 subunits could be due to the treatment of our samples and not to polyubiquitination. We therefore constructed two N-terminally epitope tagged α subunits, FNα1 and FNα2, and specifically immunoprecipitated the two proteins with a monoclonal antibody against the influenza virus hemagglutinin epitope, 12CA5. Introduction of the epitope tag does not noticeably interfere with the function of the Na,K-ATPase α subunit [21].

As shown in Fig. 2Ai, the antibody 12CA5 immunoprecipitates a heterogeneous mixture of α1 subunits from COS cells expressing FNα1. In this particular experiment the 100 kDa unmodified form of the FNα1 subunit does not constitute a

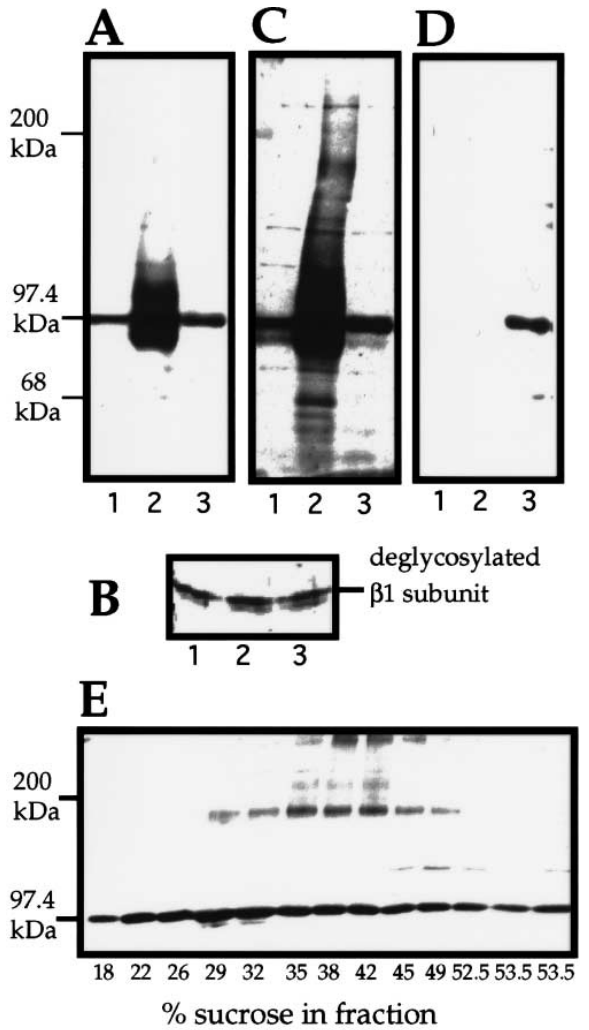

Fig. 1. Immunoblots of COS-7 membrane preparations. Electrophoresis and immunoblotting were carried out as described in Section 2. The immunoblots shown in A–D represent data from a single set of transfection experiments. Crude membranes were prepared from COS-7 cells transfected with pMT-2 (lane 1); pKC-4 α1 (lane 2) and pMT-2 α2 (lane 3) at 3 days post transfection as described in Section 2. A: Immunoblot probed with the α1 subunit specific polyclonal antibody 620. 15 μg of crude membranes were loaded per lane. B: Immunoblot probed with the β1 subunit specific antibody, SPETβ1 [16]. To facilitate detection of the COS-7 β1 subunit, 25 μg of crude membranes per lane were deglycosylated as described in Section 2. C: Longer exposure of immunoblot shown in A. D: Immunoblot probed with the α2 specific antibody 164. 15 μg of crude membranes was loaded per lane. E: Immunoblot of fractions collected from a discontinuous sucrose gradient probed with the α2 specific antibody 164. Crude membranes prepared from COS-7 cells transfected with pMT-2 α2 were fractionated on a discontinuous sucrose gradient as described in Section 2. The % sucrose present in each fraction is shown below the corresponding lane of the immunoblot.

majority of the mixture of FNα1 subunits that was immunoprecipitated. As shown in Fig. 2Aii, the higher molecular weight forms of the α1 subunit are also recognized by an antibody against ubiquitin, indicating that they are polyubiquitinated forms of the α1 subunit. FNα1 subunits conjugated to shorter polyubiquitin chains are probably not visible at this exposure, because both molecular weight and the intensity of immunostaining are proportional to the number of ubiquitin molecules that have been attatched to the FNα1 subunit.

In Fig. 2B we confirm that the higher molecular weight forms of the α2 subunit are also polyubiquitinated. In 12CA5 immunoprecipitates from COS-7 cells expressing FNα2, the α2 specific antibody 164 recognizes a prominent band at 100 kDa and a much fainter population of heterogeneous high molecular weight population of FNα2 subunits (Fig. 2Bi). Ubiquitin immunostaining clearly correlates with the heterogeneous high molecular weight population of FNα2 subunits (Fig. 2Bii).

Clearly the rat α1 and α2 subunits are ubiquitinated in COS-7 cells. While it is possible that ubiquitination of the α subunit is an artifact of COS-7 expression, we feel this is

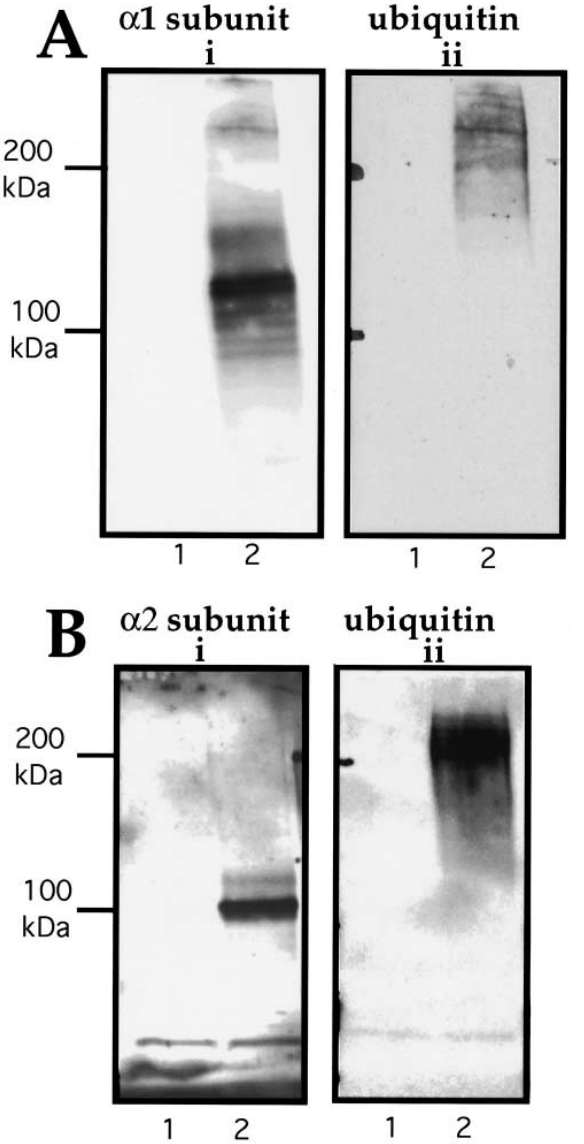

Fig. 2. Immunoprecipitation of epitope tagged α1 and α2 subunits. Lysates of COS-7 cells expressing the N-terminally epitope tagged α subunits, FNα1 and FNα2 were prepared as described in Section 2. The cDNAs for the N-terminally epitope tagged α subunits were constructed by fusing the sequence of the influenza virus hemagglutinin epitope (YPDVPDYA) to their N-termini as described in Section 2. The epitope tagged α subunits were immunoprecipitated with the monoclonal antibody 12CA5 and immunoblotted as described in Section 2. Immunoblots of 12CA5 immunoprecipitates of COS-7 cells transfected with (A) pMT-2 (lane 1) and pKC-4 α1 (lane 2) or (B) pMT-2 (lane 1) and pMT-2 α2 (lane 2) are shown. The immunoblots were probed with (Ai) the α1 specific antibody 620, (Bi) the α2 specific antibody 164, or (Aii and Bii) the ubiquitin specific antibody of Haas and Bright [17].

unlikely. The electrophoretic mobility of the α2 subunit in HeLa cells is also consistent with polyubiquitination (data not shown).

To our knowledge, this is the first demonstration that the Na,K-ATPase α subunit is ubiquitinated. There are several possible functions for the ubiquitination of the α subunit. One possibility is that polyubiquitination plays a role in the degradation of unassembled α subunits that are retained in the endoplasmic reticulum. Misfolded lumenal endoplasmic reticulum proteins and a type I integral membrane protein, MHC class I, have been shown to be extruded from the endoplasmic reticulum, apparently by the same machinery which catalyzes the insertion of proteins into the endoplasmic reticulum [14]. In the cytosol, these proteins are subsequently polyubiquitinated and degraded by the proteasome [14]. In contrast, polyubiquitinated forms of CFTR, a polytopic membrane protein like the Na,K-ATPase α subunit, remain associated with the endoplasmic reticulum membrane [30,31]. The cytosolic domains of misfolded CFTR molecules are ubiquitinated, presumably by an integral endoplasmic reticulum membrane ubiquitin conjugating enzyme such as the UBC6 protein of yeast [32], and degraded by the proteasome. There is as yet no evidence that the CFTR is translocated back across the endoplasmic reticulum membrane.

As shown in Fig. 1E, we have obtained evidence which suggests that polyubiquitination may indeed play a role in the endoplasmic reticulum associated degradation of unassembled α2 subunits. In this fractionation experiment, the immunostaining of the 100 kDa of the α2 subunit peaks between 29% and 32% sucrose, a position consistent with a plasma membrane localization (data not shown). In contrast, the high molecular weight, presumably polyubiquitinated forms of the α2 subunit peak between 38 and 42%, a density consistent with localization in the endoplasmic reticulum. Clearly, further experimentation is required to confirm this hypothesis.

Polyubiquitination of the α subunit may also play a role in the removal of excess and inactive Na,K-ATPase molecules from the cell surface. Polyubiquitination has been linked to the endocytosis and degradation of a number of plasma membrane proteins [13,15]. In the case of a truncated form of Ste2p, a yeast G-protein linked receptor, ubiquitination has been shown to be required for its ligand-stimulated endocytosis [15]. The lysosome has also been shown to play an important role in the degradation of many polyubiquitinated plasma membrane proteins [13,15]. Since there is evidence that ouabain bound to Na,K-ATPase molecules at the cell surface is internalized and degraded in the lysosome [33,34], it is likely that ubiquitination of the Na,K-ATPase may be involved in this process.

In conclusion, we have demonstrated that the α1 and α2 subunits of the Na,K-ATPase are polyubiquitinated in COS-7 cells. We propose that polyubiquitination may be involved in regulating the number of Na,K-ATPase molecules in two ways: by promoting the endoplasmic reticulum associated degradation of unassembled and misfolded α subunits, and by participating in the internalization and subsequent degradation of cell surface Na,K-ATPase molecules. Thus the cell maintains a plasma membrane population of functional Na,K-ATPase molecules which is able to sustain the sodium and potassium gradients essential for viability.

Acknowledgements: We wish to thank Dr. Kyunglim Yoon for constructing and sharing the epitope tagged α subunits. We would also like to thank Dr. Steven Smith for generously providing us with both the ubiquitin antibody and technical assistance. In addition, we are grateful to Dr. Pablo Martin-Vasallo for furnishing us with the β1 specific antibody. Finally, we wish to acknowledge the rest of the Guidotti laboratory, especially Lorraine Santy, for their assistance, input, and encouragement. This work was supported by Grants DK27626 and HL08893 from the NIH. M.V.C. was partially funded by NIH Training Grant GM07598.

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