Topology of NAT2, a Prototypical Example of a New Family of Amino Acid Transporters

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Amino acids are the predominant form of nitrogen available to the heterotrophic tissues of plants. These essential organic nutrients are transported across the plasma membrane of plant cells by proton-amino acid symporters. Our lab has cloned an amino acid transporter from Arabidopsis, NAT2/AAP1, that represents the first example of a new class of membrane transporters. We are investigating the structure and function of this porter because it is a member of a large gene family in plants and because its wide expression pattern suggests it plays a central role in resource allocation. In the results reported here, we investigated the topology of NAT2 by engineering a c-myc epitope on either the N or C terminus of the protein. We then used in vitro translation, partial digestion with proteinase K, and immunoprecipitation to identify a group of oriented peptide fragments. We modeled the topology of NAT2 based on the lengths of the peptide fragments that allowed us to estimate the location of protease accessible cleavage sites. We independently identified the location of the N and C termini using immunofluorescence microscopy of NAT2 expressed in COS-1 cells. We also investigated the glycosylation status of several sites of potential N-linked glycosylation. Based on the combined data, we propose a novel 11 transmembrane domain model with the N terminus in the cytoplasm and C terminus facing outside the cell. This model of protein topology anchors our complementary investigations of porter structure and function using site-directed and random mutagenesis.

Amino acids are actively transported into plant cells by proton-coupled symporters (1). These proteins link translocation across the plasma membrane to the proton-motive force generated by a P-type, H\(^{+}\)-ATPase (2, 3). In plants, there are many heterotrophic tissue systems that are dependent upon carbon and nitrogen import for growth and development. Since amino acids are the primary form of nitrogen available to the heterotrophic plant tissues, the amino acid symporters are responsible for the systemic distribution of organic nitrogen, and therefore, are essential contributors to plant growth (4, 5). Detailed investigations of the transport properties and bioenergetics of these symporters using isolated plasma membrane vesicles and imposed proton electrochemical potential differences have shown that they are electrogenic transporters that are driven by either transmembrane proton or electrical potential differences (6). These transporters are inhibited by chemical modification of histidine residues by diethyl pyrocarbonate (6), and substrate protection experiments suggest the sensitive residue is at or near the substrate binding site (5, 7). Several classes of symporters were initially resolved based on expression patterns and substrate specificity (6, 8–11). Transport competition experiments showed that binding sites are stereo-specific and identified the carboxylic acid, the alpha amino group, and substitutions at the \(\beta\)-carbon as important determinants in governing substrate binding (8, 9). Recently, one of these symporters was expressed in Xenopus oocytes, and electrophysiological methods allowed for a high resolution investigation of transport kinetics that suggests these transporters operate by a simultaneous binding mechanism (12).

The first plant amino acid symporter cloned (NAT2/AAP1) was identified by two groups using functional complementation of yeast amino acid transport mutants with different Arabidopsis cDNA expression libraries (13, 14). The deduced amino acid sequence of the encoded protein contains 485 amino acid residues with a calculated molecular mass of 52.9 kDa and three sites of potential N-linked glycosylation. Hydropathy analysis suggested this is an integral membrane protein with 10–12 membrane-spanning regions. A search of the non-redundant protein data bases did not identify any strong homologies, suggesting NAT2/AAP1 represented a new class of transport protein (13).

Several amino acid transporter genes have now been isolated from Arabidopsis using functional complementation of yeast transport mutants (15). These include five clones that are closely related to NAT2/AAP1 (AAP2–6) (16–18), a cationic amino acid transporter (AAT1) (19), two proline transporters (ProT1 and ProT2) (18), and a lysine and histidine transporter (LHT1) (20). Most of the plant amino acid transporters have relatively broad substrate specificity although they often exhibit some preference (lower \(K_m\) or higher \(V_{\text{max}}\)) for related groups of amino acids. The presence of multiple genes coding for amino acid carriers suggests there is considerable complexity in the function of these transporters in nitrogen allocation in plants (21).

Despite the physiological importance of amino acid transporters in plant growth, little is known about these transport proteins at the molecular level. This deficiency is a result of the difficulty of working with low abundance membrane proteins and the apparent toxicity of these eukaryotic membrane proteins expressed in E. coli.\(^1\) To learn more about the molecular structure and function of plant amino acid symporters, our laboratory has chosen NAT2/AAP1 as a prototypical example for detailed analysis. We chose this symporter because it is a

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\(^1\) T.-J. Chiou and D. R. Bush, unpublished data.
member of a large family of translocators, because it transports amino acids that are commonly found in the phloem translocation stream, and also because it is widely expressed in plant tissues, suggesting it plays an important role in nitrogen partitioning. In the results reported here, we have investigated the topology of NAT2 in the plasma membrane as an important first step in describing plant amino acid symporters at the molecular level. We determined its membrane topology by engineering a c-myc epitope onto the N or C terminus, and then we expressed the chimeric proteins in a cell-free translation system, in yeast, and in COS-1 cells.

**EXPERIMENTAL PROCEDURES**

**Chimeric Gene Constructs—Arabidopsis NAT2 cDNA was digested with EcoRI, and the 1.7-kb fragment was subcloned into pBSk(+) vector under the T7 promoter to produce plasmid pBS-NAT2. An NdeI site was engineered at the nucleotide 1530 of NAT2 cDNA by site-directed mutagenesis. The site-directed mutagenesis was carried out according to the Bio-Rad manual (No. 170–3581) based on the method of Kunkel (22). The in vitro synthesis of the mutant DNA strand was performed by using the uracil-containing single-stranded DNA from plasmid pBS-NAT2 grown in Escherichia coli strain CJ236 as template with TNT coupled transcription/translation (P-5-TCCAGAGATCATAGAC-3') as primer. The pBS-NAT2/NdeI plasmids were transformed into E. coli strain DH5a (Life Technologies, Inc.), and the transformants were screened on the ampicillin LB plates and confirmed by DNA sequencing. A human c-myc epitope cassette was acquired that contains six repeats of MEKQKISEEDLNQ (the epitope is underlined). This epitope is recognized by monoclonal antibody, Myc1–9E10 (23).

**Topology of NAT2 in the Plasma Membrane**—In the results reported here, we have investigated the topology of NAT2/AAPI from A. thaliana. The NAT2/AAP1 from A. thaliana was expressed in a cell-free translation system, in yeast, and in COS-1 cells. The strain of E. coli used in this study was JT16 (pCMV5-6N) containing 6xHis epitope on the C terminus of NAT2, the 6xHis epitope cassette was amplified by polymerase chain reaction with two primers, 5'-ATCGGATTTAACATATTG-3' and 5'-GGGGTATTCTAGATCAAGT-3' (the epitope cassette in the vector pGEM7z+) was kindly provided by Dr. N. Raikhel (Michigan State University). To construct the myc epitope on the C terminus of NAT2, the 6x myc cassette was amplified by polymerase chain reaction with two primers, 5'-ATCGGATTTAAACATATTG-3' and 5'-GGGGTATTCTAGATCAAGT-3' which contain an NdeI site and an XhoI site, respectively. The NdeI/XhoI polymerase chain reaction fragment was subcloned into pBS-NAT2/NdeI by NdeI and XhoI to produce plasmid pBS-6C. To construct the myc epitope onto the N terminus of NAT2, an EcoRI site was engineered at the nucleotide position 89 on NAT2 cDNA of pBS-NAT2 by site-directed mutagenesis. The site-directed mutagenesis was performed with the primer 5'-CTCATCATTAGTTACATATTG-3' and 5'-GGGGTATTCTAGATCAAGT-3' as primer. The EcoRI fragment of NAT2/EcoRI mutant was subcloned into pGEM7z+ with 6x myc cassette on the 5'-end of the epitope cassette to produce plasmid pGEM-6N.

**Proteinase K Digestion and Immunoprecipitation—**The in vitro transcription and translation reactions were carried out in the Promega TNT reticulocyte lysate coupled system, with or without microsomal membranes, according to the manufacturer instructions. Microsomes were isolated from hen oviducts according to the method described by Lively and Walsh (30). Briefly, the magnum portion of chicken oviduct was homogenized, and the membrane fraction was separated by step sucrose gradient in Beckman SW-28 rotor for 16 h at 100,000 × g. The band between the 1.5 and 2.0 M sucrose layers was collected and treated with 15 mM EDTA. The EDTA-treated membranes were pelleted and then resuspended in 20 mM HEPES, pH 7.5, 0.2 mM sucrose, and 2 mM dithiothreitol to a concentration of 50–70 A280 units/ml, and aliquots were stored at −80 °C. For each in vitro transcription/translation reaction, 1 μg of pBS-NAT2, pBS-6C, or pGEM-6N was added. The reticulocyte lysate, amino acids, T3 or T7 RNA polymerase, [35S]methionine and [35S]cysteine (Trans-35S-label, ICN Radiochemicals), and EDTA-treated rough microsomes were added according to the manufacturer instructions. After incubation at 30 °C for 90 min, the reaction was terminated, and the products were ready for further analysis. Alkaline extraction was used to show the porter was incorporated in the microsomes. An equal volume of 0.2 mM sodium carbonate (pH 11.0) was added to the finished in vitro translation reactions and incubated on ice for 30 min. Microsomes were pelleted with 150,000 × g centrifugation for 30 min, then supernatant and pellet proteins were separated with SDS-PAGE, and radioactive bands were visualized by fluorography using ENHANCE (NEN Life Science Products).

**In Vivo Labeling of COS-1 Cell Proteins and Immunoprecipitation—**In vivo transcription and translation reactions were centrifuged at 150,000 × g for 30 min. The membrane pellets were washed with TE buffer (10 mM Tris, pH 7.6, and 1 mM EDTA) and repelleted. Washed membranes were resuspended in ice-cold TE with proteinase K (20 μg/ml). Proteolysis proceeded on ice for 10–60 min in the presence or absence of 1% Triton X-100 and was stopped by adding 500 mM PMSF. 10 mM Tris-HCL, pH 7.6, 0.15 M NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride). The proteolytic peptides were collected by immunoprecipitation with Myc1–9E10 monoclonal antibody. Immunoprecipitation was carried out according to the method described by Szczenza-Skorupa and Kemper (31). After incubation with antibody overnight, 50 μl of protein A-Sepharose slurry (40 mg/ml SenthaGel, Pharmacia Biotech Inc.) was added to the immunoprecipitates and incubated for an additional hour. After washing with RIPA buffer and TSA buffer (10 mM Tris-HCL, pH 7.6, 0.15 mM NaCl, and 1 mM EDTA), immunoprecipitated proteins were eluted by heating at 65 °C for 10 min in SDS gel loading buffer. Proteins were separated on 12% gels with SDS-PAGE and visualized by fluorography.

**Transfection of COS-1 Cells—**COS-1 cells were maintained at 37 °C with 6% CO2 in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Inc.), supplemented with 10% calf serum (Sigma) and 100 units/ml penicillin and 0.1 mg/ml streptomycin (Life Technologies, Inc.). At 80% confluence, COS-1 cells were transfected in 35-mm culture dishes with the expression vector pCMV5 containing epitope-tagged NAT2 cDNAs or with pCMV5 vector only as mock cells. 0.1 ml of DMEM containing 2 μg of DNA was mixed with same amount of DMEM with 5 μl of Lipofectin (Life Technologies, Inc.) and incubated for 30 min at room temperature, and 0.8 ml of DMEM was added to the mixture and placed on COS-1 cells. After incubation for 4 h, the transfection mixture was aspirated and replaced with DMEM with 10% calf serum. Cells were grown in medium for 48 h before [35S]protein labeling or immunofluorescent staining.

**In Vivo Labeling of COS-1 Cell Proteins and Immunoprecipitation—**COS-1 cells were grown and transfected in 35-mm dishes for radiolabeling of recently synthesized proteins (31). 48 h after transfection, cells were pre-incubated for 30 min in methionine- and cysteine-free DMEM. Cells were then labeled for 2 h with the same medium containing 120 μCi/ml of [35S]methionine and [35S]cysteine (Trans-35S-label, ICN Radiochemicals). After labeling, cells were washed twice with ice-cold PBS.
Expression of myc-tagged NAT2 in Yeast—Chimeric NAT2 proteins were constructed with either an N- or C-terminal human myc epitope cassette (containing six copies of the myc epitope in series) as N-myc NAT2 and C-myc NAT2. The myc-tagged genes were subcloned into a yeast/E. coli shuttle vector, pNEV-E (24). Expression in yeast was driven by PMA1 (yeast myc) for each treatment. 

Indirect immunofluorescence was performed according to the method of Szczesna-Skorupa and Kemper (31). Briefly, COS-1 cells were grown on coverslips in 35-mm Petri dishes and transfected as described. After 48 h, cells were washed twice with PBS and fixed with 2.5% paraformaldehyde for 20 min. Cells were directly incubated with antibody or permeabilized with 0.1% Triton X-100 for 5 min followed by washing with 0.1% gelatin in PBS. Incubation with Myc1–9E10 monoclonal antibody was carried out for 40 min at room temperature followed by a 30-min incubation of a secondary antibody, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories). Cells were washed with 0.1% gelatin in PBS between incubation of antibodies. Cells were observed and photographed using Zeiss photomicroscope III equipped with epi-illumination optics and an HBO 100-watt mercury lamp.

RESULTS

Expression of myc-tagged NAT2 in Yeast—Chimeric proteins were expressed in a cell-free translation system with ER-derived microsomal membranes followed by proteolysis with 20 μg/ml of proteinase K on ice for 10–60 min in the presence or absence of 1% Triton X-100. Proteolytic products were immunoprecipitated with human Myc1–9E10 monoclonal antibody, followed with SDS-PAGE and fluorography.

Immunofluorescent Staining—Indirect immunofluorescence was performed according to the method of Szczesna-Skorupa and Kemper (31). Briefly, COS-1 cells were grown on coverslips in 35-mm Petri dishes and transfected as described. After 48 h, cells were washed twice with PBS and fixed with 2.5% paraformaldehyde for 20 min. Cells were directly incubated with antibody or permeabilized with 0.1% Triton X-100 for 5 min followed by washing with 0.1% gelatin in PBS. Incubation with Myc1–9E10 monoclonal antibody was carried out for 40 min at room temperature followed by a 30-min incubation of a secondary antibody, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories). Cells were washed with 0.1% gelatin in PBS between incubation of antibodies. Cells were observed and photographed using Zeiss photomicroscope III equipped with epi-illumination optics and an HBO 100-watt mercury lamp.

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Expression of myc-tagged NAT2 in a Cell-free Translation System and Partial Proteolysis—To study the topology of NAT2, the epitope-tagged proteins were expressed in vitro and investigated with partial proteolysis and immunoprecipitation. The myc-tagged NAT2 proteins were expressed in a cell-free translation system in the presence of microsomes. Alkaline extraction of the microsomes after in vitro translation showed that >50% of the translated protein was incorporated in the microsome membranes (data not shown). After in vitro cotranslation, samples were treated on ice with 20 μg/ml proteinase K for 10–60 min, and then the proteolytic fragments were collected by immunoprecipitation with Myc1–9E10 monoclonal antibody. The precipitated peptides were separated with SDS-PAGE and visualized by fluorography (Fig. 2A).

The rationale behind this experiment is that hydrophilic loops of NAT2 protein that are on the cytoplasmic side of the microsomal membrane are exposed to the protease while transmembrane domains and peptide loops on the luminal side of the microsome are protected from proteolysis. The N-myc NAT2 was not detected after 10 min of proteolysis, suggesting the N-terminal epitope was degraded by proteinase K (Fig. 2A). The resulting proteolytic fragments could not be immunoprecipitated with anti-myc antibody because they lacked the N-terminal myc epitope. These results are consistent with the N terminus of NAT2 facing the cytoplasmic side of the microsome. In contrast, the C-terminal myc epitope was not sensitive to proteinase K, suggesting the C terminus of NAT2 was located on the luminal side of the microsomal membrane vesicle (Fig. 2A). Moreover, proteolysis of C-myc NAT2 generated six peptide fragments of decreasing molecular mass that retained the C-terminal tag and were precipitable by the anti-myc antibody (Fig. 2A). When proteolysis was performed in the presence of Triton X-100, no protein fragments were detectable, which is...
consistent with the notion that protease-resistant peptides were protected by the membrane. The lower molecular weight products observed in the absence of proteolysis are distinct from the proteolytic fragments and may be due to internal initiation of translation. The size of the C-terminal-tagged proteolytic products enabled us to estimate the approximate cleavage sites of protease K. Since protease K is not membrane-permeable, hydrolysis is dependent on the accessibility of loop regions of the peptide that exist between adjacent transmembrane domains. Thus, band 1 represents cleavage at loop I and band 2 at loop II and so on (Fig. 2A).

Computer-generated models of NAT2 topology that consider hydrophobicity and the positive inside rule predict 10 or 11 membrane-spanning regions (32). The estimated length of the proteolytic fragments resulting from protease K digestion are consistent with the 11 transmembrane domain (TMD) model. Based on differential sensitivity of the myc epitope to proteolysis, the size and number of proteolytic fragments, and the computer prediction, we propose that NAT2 protein contains 11 membrane-spanning regions, with the N terminus in the cytoplasm and C terminus facing outside the cell (Fig. 2B).

According to our model of NAT2 topology, three sites of potential N-linked glycosylation are not on the outside face of the plasma membrane (Fig. 3A). Therefore, these sites would not be exposed to the luminal side of the ER during protein synthesis and they should not be glycosylated. To test this hypothesis, NAT2 was expressed in rabbit reticulocyte lysate with or without ER-enriched microsomal membranes. The expressed proteins were separated with SDS-PAGE and visualized by fluorography. Without microsomes, the molecular mass of NAT2 on SDS-PAGE was about 45 kDa, and yeast α-factor, a glycoprotein, was about 20 kDa.

PAGE in the presence or absence of microsomes, suggesting it was not glycosylated (Fig. 3B). As a positive control, the yeast α-factor exhibited a mobility shift due to glycosylation when co-translated with microsomes (Fig. 3B). The absence of glycosylation for NAT2 supports the 11 transmembrane domain model.

Expression and Immunofluorescent Staining in COS-1 Cells.—To test the 11 transmembrane domain model independently, we expressed myc-tagged NAT2 proteins in COS-1 cells. The cells were labeled with [35S]methionine/cysteine and then immunoprecipitated with c-myc antibody followed by SDS-PAGE and fluorography (Fig. 4). Both N- and C-terminal tagged proteins exhibited the same molecular weight as they did in the in vitro system, thereby demonstrating their expression in COS-1 cells. An extra, high molecular weight band was observed with the N-myc NAT2. This may reflect a mobility shift due to an unknown factor interacting with the cytoplasmic c-myc epitope or modification of the N-myc NAT2 product. The presence of this band was insensitive to hydrolysis by endoglycosidase H and N-glycosidase F (not shown), suggesting glycosylation was not involved.

The transfected cells were used for indirect immunofluorescent labeling with FITC-conjugated goat anti-mouse IgG. Cells were incubated in the presence or absence of Triton X-100 to compare intracellular and cell surface staining patterns. In the permeabilized mock cells, some background staining can be seen in nuclei (Fig. 5B), which may be due to the endogenous c-myc protein. Fluorescence was visible in the internal membranes (i.e. endoplasmic reticulum and Golgi) and plasma membrane in both of the permeabilized N- and C-terminal tagged NAT2 transfected cells (Fig. 5, D and F). For the unpermeabilized cells, however, only the C-terminal epitope-tagged protein was detected on the cell surface (Fig. 5E), indicating the C terminus is oriented on the outside of the plasma membrane. In contrast, the N-terminal epitope was not detected in unpermeabilized cells (Fig. 5C). These data provide additional evidence that the N terminus of NAT2 is inside the cell and the C terminus is outside. Thus, the results of immunofluorescent staining in COS-1 cells further support the 11 transmembrane domain model.

DISCUSSION

In the results presented here, we showed that the N terminus of NAT2/AAP1 is on the cytoplasmic side of the plasma.
membrane, and the C terminus faces outside the cell. This conclusion was supported by the differential sensitivity to proteolysis by the N- and C-terminally tagged NAT2 proteins and by immunofluorescent localization of epitope-tagged NAT2 in COS-1 cells. In addition, partial proteolysis of the in vitro translated C-terminal tagged protein produced six immunoprecipitable peptide fragments, suggesting NAT2 has six protein domains that are accessable to proteinase K. An 11-TMD model of NAT2 is proposed based on the number and size of the proteolytic fragments, predictions derived from hydropathy analysis, the absence of protein glycosylation, and localization of the N and C termini of NAT2 on opposite sides of the plasma membrane. Taken together, we believe these data provide good evidence that NAT2 contains 11 TMD.

The novel 11 transmembrane domain model we propose here is different from the well-known model of the major facilitator superfamily that contains a common structural motif of 12 transmembrane segments with cytoplasmic N and C termini (34–37). This superfamily contains many plasma membrane carriers in this family exhibit very similar hydrophobic profiles and inorganic substrates (34–39). NAT2 is also unique when compared with other Arabidopsis amino acid transporters such as ProT1, ProT2 (proline transporter; Ref. 18), and AAP1 (cationic amino acid transporter; Ref. 19). So far, the 11-transmembrane topology is specific to the Arabidopsis AAP family.

An alignment of the hydropathy profile of the AAP family members shows that they are super-imposable (32). This observation supports our focus on NAT2 as a prototypical example of this family. Interestingly, there is a hyper-variable region between TMD5 and TMD6 of the alignment. According to the topology model, this region is oriented to the outside of the cell. This observation raises the interesting hypothesis that this loop may be involved in defining substrate specificity. When we compare the amino acid sequences in this region, some members contain similar numbers of charged amino acid residues. For example, AAP2 and AAP4 have three basic amino acids, and they transport the same group of amino acids. Likewise, AAP3 and AAP5 share significant similarities in both charge and substrate patterns (17, 18). The importance of these observations will require additional investigations.

As a growing number of amino acid transporters are identified in higher plants, it becomes increasingly important to define the molecular characteristics of these amino acid transporters in the context of nitrogen allocation across the plant as a multicellular organism. The topology of NAT2/AAP1 described here is the foundation of future investigations of the structure and function of the Arabidopsis AAP family.

Acknowledgments—We thank Dr. N. V. Raikhel (Michigan State University, East Lansing, Michigan) for providing the 6x-myc cassette, Dr. E. Szczesna-Skorapa and Ci-Di Chen (Department of Molecular and Integrative Physiology, University of Illinois) for discussing the in vitro transcription/translation experiments and help with COS-1 cell transfection and indirect immunofluorescence.

REFERENCES

1. Bush, D. R. (1993) Annu. Rev. Plant Physiol. Plant Mol. Biol. 44, 513–542
2. Briskin, D. P. (1990) Biochem. Biophys. Acta 1019, 95–109
3. Serrano, R. (1989) Annu. Rev. Plant Physiol. Plant Mol. Biol. 40, 61–94
4. Pate, J. S. (1983) in Plant Physiology: A Treatise, Vol. VIII: Nitrogen Metabolism. (Stewart, F. C. and Bidwell, R. G. S., eds) pp. 335–401, Academic Press, New York
5. Bush, D. R. (1998) in Plant Amino Acids: Biochemistry and Biotechnology. (Singh, B., ed) Marcel Dekker, NY, in press
6. Li, Z.-C., and Bush, D. R. (1992) Plant Physiol. 96, 268–277
7. Bush, D. R., and Li, Z.-C. (1992) in Recent Advances in Plasmid Transport and Assimilate Compartmentation. (Bonemain, J. L., Delrot, S., Lucas, W. J., and Dainty, J., eds) pp. 148–153, Presses Académiques, France
8. Li, Z.-C., and Bush, D. R. (1991) Plant Physiol. 96, 1338–1344
9. Li, Z.-C., and Bush, D. R. (1992) Arch. Biochem. Biophys. 294, 519–526
10. Williams, L. E., Nelson, S. J., and Hall, J. L. (1992) Plant Physiol. 100, 541–550
11. Williams, L. E., Nelson, S. J., and Hall, J. L. (1990) Planta 180, 540–545
12. Boorer, K. J., Frommer, W. B., Bush, D. R., Kremen, M., Lee, D. F. P., and Wright, E. M. (1996) J. Biol. Chem. 271, 2123–2220

3 L. Chen and D. R. Bush, unpublished data.
