Primary Structure and Differential Expression during Development and Pregnancy of a Novel Voltage-gated Sodium Channel in the Mouse*

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Until recently, all cloned vertebrate voltage-dependent sodium channels exhibited high sequence homology to one another and appeared to comprise a single multi-gene subfamily. An exception is the human Na,,2.1 channel proposed to represent a second Na+ channel (NaCh) gene subfamily since comparison with previously cloned voltage-gated NaChs revealed only 40–45% identity. We have now cloned a mouse NaCh (mNa,2.3) from an atrial tumor cell line that shows high amino acid sequence identity to hNa,2.1 in functionally relevant regions such as the pore-forming segments, S4 segments, and inactivation gate sequence. Overall sequence identity is 68%. mNa,2.3 mRNA was most abundant in heart and uterus, and the transcript levels in heart, brain, and skeletal muscle were differentially regulated during development. Transcript levels in heart were greatest immediately after birth. mNa,2.3 transcript levels in pregnant uterus increased 3-fold between day 15 of pregnancy and birth and then declined 15-fold during the 2 days following delivery. The mNa,2.3 amino acid sequence indicates that the Na,2 NaCh gene subfamily is well conserved across species, and the tissue-specific and developmental regulation of mRNA expression suggests these channels play important physiological roles in cardiac and uterine muscle.

Voltage-dependent sodium channels (NaChs) are responsible for the depolarizing phase of most nerve and muscle action potentials and are essential for many diverse physiological processes (1–3). Six full-length NaCh cDNA sequences have been cloned from mammalian brain (4–6), heart (7–9), and skeletal muscle (10–12). All but one of these reported cDNA sequences predict proteins that exhibit striking similarity to one another (>60% overall amino acid sequence identity) and appear to comprise a single multi-gene family. This mammalian NaCh gene family has related members in such diverse species as eel (13), Drosophila (14, 15), jellyfish (16), and squid (17), with the eel NaCh being most similar to the mammalian proteins. The mammalian NaCh cDNA clone that is least related to the group of similar mammalian channels is the hNa,2.1 protein cloned from human heart (9). This protein shows less than 50% overall identity with other NaChs and contains unique amino acid sequence in functionally important regions that are well conserved among all other vertebrate NaChs. Hence, the hNa,2.1 NaCh has been placed in a second NaCh gene subfamily designated Na,2, while all previously described NaChs can be placed in the Na,1 subfamily. The hNa,2.1 channel was the first cloned NaCh to suggest multiple NaCh gene subfamilies exist and that NaCh diversity may approach that seen with K+ channels.

While the hNa,2.1 protein offers a wealth of structure function information due to its unusual amino acid sequence in functionally important regions, it has not been functionally expressed despite extensive efforts utilizing the Xenopus oocyte, Chinese hamster ovary cell, and HEK 293 cell heterologous expression systems. This lack of functional expression has called into question the physiological relevance of this single human cDNA clone. While expression failure could be explained by the requirement for additional subunits or specialized cellular biosynthetic machinery, other explanations include cloning artifacts or that the hNa,2.1 clone represents an expressed pseudo-gene. However, the identification of a putative Na,2 subfamily member in the rat strengthens the argument that this unusual channel is physiologically relevant. A partial cDNA sequence encoding the C-terminal one quarter of a rat glial NaCh has been reported that shows 78% sequence identity with the human Na,2.1 NaCh and less than 50% identity with other vertebrate NaChs (18), suggesting the Na,2 gene subfamily exists in nonhuman species. Since this partial clone represents the second reported member of the Na,2 subfamily, it is classified best as rat Na,2.2. However, since it was originally referred to as a glial NaCh, this designation will be used in this paper.

We report here the cloning of a full-length cDNA from the mouse AT-1 atrial tumor cell line (19–21) that encodes a mammalian NaCh isoform representing the first member of the Na,2 family in the mouse. This NaCh has been designated mNa,2.3, in keeping with our earlier nomenclature (9). mNa,2.3 is overall 68% identical to hNa,2.1 with similar sequence in functionally relevant regions such as pore-forming

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1 The abbreviations used are: NaCh, voltage-dependent sodium channel; RACE, rapid amplification of cDNA ends; MOPS, 4-morpholinepropanesulfonic acid; PCR, polymerase chain reaction.

2 T. J. Knittle, M. M. Tamkun, and P. B. Bennett, unpublished results.

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**EXPERIMENTAL PROCEDURES**

**Materials**—[α-32P]dATP (3000 Ci/mmol) was purchased from ICN (Griva, CA). PC-1 medium was obtained from Hycor Biomedical Inc. (Portland, ME). Fetal bovine serum, penicillin, streptomycin, and the Taq polymerase were purchased from Promega. All other chemicals and reagents were of analytical grade.

**Animals**—DBN2J male and C57BU6J female mice (Jackson Laboratory) and their F1 were used. They were mated at 60-65 days of age. Impregnation was assessed by the presence of either spermatozoa in the vaginal smear or vaginal plug. That day was designated as day 0 of pregnancy, and the delivery was on day 19. While pregnancy studies were done on C57BU6J female mice, developmental and tissue-specific experiments were performed on C57BL/6J X DBA/2JF, animals.

**AT-1 Cell Culture**—AT-1 cells were kindly provided by Loren Field (Kranert Institute, Indianapolis, IN). Cells were subconfluent into [C57BL/6 X DBA/2JF] female mice. In 6–8 weeks, subcutaneous tumors appeared obvious, and the cells were isolated 12–16 weeks after inoculation following a method adapted from previously described methods (19, 21). Briefly, tumor-bearing mice were killed, and the tumor tissue was obtained with phosphate-buffered saline (0.9% NaCl, 10 mM NaH2PO4, pH 7.0), finely minced, and placed for 1 h at 37 °C with gentle rocking in phosphate-buffered saline containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.1% collagenase. The cell suspension obtained was sedimented, washed with phosphate-buffered saline, resuspended in the PC-1 medium, supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), dexamethasone (10 μM), and fetal bovine serum (10%), and plated at a density of 250–325 × 106 cells/ml. The medium was changed every other day, and the cultures grew to confluence and were usually beating spontaneously within 10–14 days in culture.

**Total RNA Extraction, Poly(A)+ RNA Purification, and Northern Blot Analysis**—Total RNA was isolated using the guanidinium thiocyanate method, and poly(A)+ RNA was purified through oligo(dT)-cellulose chromatography by standard procedures (3, 22). For Northern blot studies, up to 50 μg of total RNA were fractionated by electrophoresis through a 1% agarose, 3% formaldehyde gel in 20 mM MOPS, 1 mM EDTA, pH 7.4. Application of equal amounts of RNA to each lane was confirmed by the ethidium bromide to the samples prior to electrophoresis and the quantification of the stained ribosomal bands by means of a imaging densitometer (Bio-Rad). Less than 10% variability was observed among lanes. The gel was submerged for 5 min in 50 mM Na2CO3 and 1.5 mM NaCl and then neutralized 30 min in 1 x Tris, pH 6.8, and 1.5 mM NaCl before an overnight transfer to a Nytran filter (Schleicher & Schuell) by capillary action in 20 ul SSC and 1% SDS before autoradiography. The quantitation of the regions encompassed by each band was determined by densitometry with a Molecular Dynamics. At least three different filters made from pooled samples were analyzed in each experiment. Every pool had RNA levels in uterus during pregnancy vary and dramatically decrease following delivery. Taken together, these data indicate that the Na2,2 subfamily is conserved across species and likely to play important physiological roles in cardiac and uterine muscle.

**RESULTS AND DISCUSSION**

**Cloning of the mNa2,3 Channel from Mouse AT-1 Cells**—A mouse atrial tumor cell line (AT-1) was chosen for this work since the study of ion channels is simplified in cell lines where individual cells can be studied by voltage clamp and populations studied with cellular and molecular techniques. AT-1 cells, derived from atrial tumors in transgenic mice carrying fusions between the atrial natriuretic factor promoter and the SV40 large T antigen coding sequence, retain a highly differentiated cardiac phenotype in culture, including expression of adult cardiac-specific proteins, spontaneous beating, and secretion of atrial natriuretic factor (19, 21). A cDNA library was constructed from a beating AT-1 cell preparation and screened at low stringency with hNa2,2-derived probes as described under "Experimental Procedures." Four cDNA clones were isolated and sequenced as shown in Fig. 1. The longest clone (C171) was 5 kilobases in length and contained 75% of the coding sequence. Repeated screening of the library with 5'-fragments of the C171 clone failed to isolate additional sequence, necessitating the use of 5'-RACE-PCR. Two successive rounds of RACE produced the two fragments listed as RCT15.
cDNAs encoding the mNa2,3 clone. The bar represents the longest open reading frame encoded by these overlapping cDNAs, and untranslated regions are shown by the ranking black lines. Clones C291, C151, C171, and C191 were isolated from the AT-1 cDNA library. Clones RC715 and RB1401 were obtained by 5′-RACE using AT-1 cell first strand cDNA as described under “Experimental Procedures.”

Fig. 1. cDNAs encoding the mNa2,3 clone. The bar represents the longest open reading frame encoded by these overlapping cDNAs, and untranslated regions are shown by the ranking black lines. Clones C291, C151, C171, and C191 were isolated from the AT-1 cDNA library. Clones RC715 and RB1401 were obtained by 5′-RACE using AT-1 cell first strand cDNA as described under “Experimental Procedures.”

and RB1401 in Fig. 1. All cDNA fragments were sequenced on both strands, and the sequence of PCR products from separate amplifications was compared to guard against polymerase-induced sequence error.

The complete sequence, designated mNa2,3, consists of 251 base pairs of 5′-untranslated sequence, an open reading frame of 5043 base pairs, and at least a 2.0-kilobase 3′-untranslated region. A polyadenylation signal sequence (AATAAA) and a poly(A) tail were not found. The nucleotide sequence immediately surrounding the assigned initiation codon (CGAAAATGT, nucleotides 247–254) resembles the consensus eukaryotic initiation sequence (23); only in the presence of a cytosine nucleotide at position −5 and an adenosine nucleotide at position −3 (relative to the start codon). Two in-frame termination codons (TAA and TGA) are present starting at nucleotide 5295. Interestingly, 5′ of the assigned start codon in previously cloned vertebrate NaChs belonging to the Na1 family is an out of frame ATG triplet at position −8 relative to a typical eukaryotic initiation sequence (ATGCCAACATGG). However, in the human Na2,1 sequence, while this motif was present 5′ to the assigned initiation codon, both ATGs preceded short open reading frames. The mNa2,3 channel lacks the upstream ATGs, and the first in frame methionine codon corresponds to the predicted translation start site in the human Na2,1 channel. No other potential translation start sites exist within the 5′-untranslated region.

The deduced amino acid sequence of the AT-1-derived sequence is shown in Fig. 2 and compared with the full-length human Na2.1 (9), H1 from rat heart (8), and the rat glial partial sequence (18). The deduced primary structure of mNa2,3 consists of 1861 amino acids and has a calculated molecular weight of 192,144. Prediction of transmembrane topology by hydropathy analysis reveals a profile similar to other NaChs, with four large (229–290 residues) hydrophobic domains, each composed of at least six potential membrane spanning α-helical segments including a positively charged amphipathic segment (S4). As with the original hNa2.1, mNa2,3 shares no significant overall amino acid identity with voltage-dependent potassium channels or other known protein sequences. The amino acid identity with voltage-dependent Ca2+ channels is less than 10% (24). Comparison of mNa2,3 with each of the five complete rat NaCh sequences reveals a uniform pattern of overall primary structure homology (overall amino acid identity is 39% for rat brain I (4), 34% for rat brain II (4), 33% for rat brain III (5), 33% for rat skeletal muscle SM1 (11), and 33% for rat heart I (8)). However, mNa2,3 is more homologous to hNa2,1 (68% amino acid sequence identity) than it is to these rat channels, indicating a high degree of relatedness to Na2 subfamily of NaChs. Significant homology is also evident from comparisons of mNa2,3 with NaChs from the eel electric organ (31% amino acid identity) (13), the Drosophila para locus (22%) (15), and squid (22%) (17). Only 15% identity was observed between mNa2,3 and the jellyfish NaCh (16).

Regional comparisons of mNa2,3 with other NaChs reveal a high degree of homology within the repeat domains where up to 84% amino acid identity is observed (Table I). In contrast, there is poor conservation of primary sequence within the interdomain regions ID1–2 and ID2–3. Clearly, the mNa2,3 channel is most similar to the hNa2,1 clone than other mammalian channels. A high degree of amino acid sequence identity (~74% when comparing mNa2,3 and rat heart I) with other NaChs is found within two short segments (SS1 and SS2) of the S5–S6 interhelical region (Fig. 2) that are believed to form membrane-penetrating hairpin structures that contribute to the formation of the ion pore (25) and various neurotoxin binding sites (26, 27).

The primary sequence of mNa2,3 shares distinct features with hNa2,1 in regions known to be important in voltage-dependent activation and inactivation. The S4 segments of these two channels, which may function as voltage-sensors, collectively exhibit fewer positive charges than is typical of other NaChs, vertebrate or invertebrate (Fig. 2). The greatest differences occur in domain 4 where there are only 4 arginine or lysine residues as compared with 8 for other NaChs. Histidines (residues 1355 and 1367) replace arginines at two positions in the S4 segment of domain 4. Other S4 segment variations include the substitution of glutamines (residues 208 and 1345) for arginines in domains 1 and 4, and aliphatic residues (Ile-1029, Leu-1349) for arginines in domains 3 and 4. The S4 segment of domain 2 in mNa2,3 has a reduced number of positively charged residues (2 instead of 5, with three arginines being replaced with leucine, valine, and isoleucine). This reduction in positive charge in the S4 segment of domain 2 represents the greatest difference between the mNa2,3 and hNa2,1 channels within functionally significant regions. All other NaChs, with the exception of the jellyfish NaCh, which lacks the fifth positive residue, contain five positive amino acids in this region. The ID3–4 region, which has an essential role in inactivation (28), is unlike the sequence found in most NaChs. The replacement of the highly conserved protein kinase C phosphorylation site (found in all previously described NaChs except jellyfish and hNa2,1) with a potential tyrosine kinase site (29) is notable. Within the putative pore-forming region, the mNa2,3 channel has amino acid substitutions at 2 residues involved in determining ionic selectivity, position 1142 is an Asn as opposed to the Lys found in all other NaChs except the hNa2,1 and jellyfish NaChs, where this position is also an Asn, and position 1454 is a Ser as opposed to the Ala found in all other NaChs except the rat glial channel.

There are five potential sites for N-linked glycosylation in regions of hNa2,1 predicted to be extracellular, while in mNa2,3 there are six sites. Like other NaChs, these potential sites are clustered in the S5–S6 interhelical regions of domains 1 and 3. Potential sites for cyclic nucleotide-dependent phos-
Cloning and mRNA Expression of a Murine NaCh

**FIG. 2.** Comparisons of mNa2.3 amino acid sequence with hNa2.1, glial NaCh, and rat heart I. Amino acid residues identical to the phosphorylation sites are indicated (*) as are possible N-linked glycosylation sites (○).

The suggested locations of the 24 putative membrane-
phorylation are also well conserved between the two channels, being present in the ID1-2 (Ser-443) and ID2-3 (Ser-756, Ser-857, Ser-869, and Ser-906) regions of mNa,2.3. A single site conserved between the two proteins in the C terminus also exists at Ser-1511. The density of these sites in the ID1-2 region is much less than in rat brain NaChs (1).

**Tissue-specific Expression of the mNa,2.3 Transcript**—Steady-state levels of mNa,2.3 transcripts were examined in various mouse tissues by Northern blot analysis as described under "Experimental Procedures" (Fig. 3). Equivalent signals were detected in RNA from the cultured AT-1 cells (lane C), heart (lane H), and uterus (lane U). Faint signals were present in RNA from brain, kidney, and skeletal muscle, but no specific hybridization was observed in RNA from liver. These results are consistent with the tissue-specific expression of mNa,2.3 primarily in heart, skeletal muscle, and uterus. This tissue specificity of expression was similar to that described for the human Na,2.1 channel (9).

Voltage-gated ion channel gene expression varies during development presumably to satisfy tissue-specific needs, and strict developmental regulation may be taken as an indication of the physiological importance of a given channel to tissue function. Therefore, the time course of expression of mNa,2.3 during development was determined in mouse heart, skeletal muscle, and brain (Fig. 4). mNa,2.3 expression increased approximately 2.5-fold in embryonic heart just prior to birth and then decreased at least 20-fold by day 7 after birth. Expression levels then slowly increased to the adult level, which was then followed by a slow decline to adult levels. Expression in brain showed a pattern similar to heart in that expression peaked just prior to birth and then declined approximately 10-fold by day 7 of life. An increase of severalfold was observed between days 7 and 14, and after day 21 the expression level increased at least 6-fold to the adult levels. Thus, the mNa,2.3 transcript is well regulated during embryonic and neonatal development in a tissue-specific manner.

Developmental regulation of both NaCh mRNA and function has been extensively studied, especially in skeletal muscle. Early in muscle development, the predominant NaCh is a TTX-resistant type represented by the heart type I isoform (8, 12, 30). Beginning several days after birth, the resistant channels are replaced by the TTX-sensitive isoform characteristic of adult skeletal muscle, and this exchange is complete within 30 days. Changes in mRNA levels parallel the developmental changes in Na⁺ currents (12, 30). Muscle NaCh transcript levels and currents are also regulated by innervation, electrical activity, cyclic AMP, and cytosolic Ca²⁺ (31–33), all of which are related. Given these examples, it is not surprising that the mNa,2.3 channel mRNA is regulated during cardiac and skeletal muscle development.

**Expression of the mNa,2.3 Transcript in Uterus during Gestation**—Since the mNa,2.3 transcript was abundantly detected in uterus, transcript expression during pregnancy was characterized as shown in Fig. 5. The mRNA levels observed at days 8 and 15 of pregnancy were equivalent to those determined in virgin tissue. However, at the two time points taken just prior to delivery, mRNA level increased 3-fold to a maximum and then dropped 15-fold within 2 days post-delivery. This increase in transcript level just prior to delivery and the precipitous drop immediately after suggest the mNa,2.3 protein is involved in priming the uterus for contraction. Voltage-gated NaCh expression in uterine smooth muscle, as followed by current amplitude during voltage clamp, does increase just prior to delivery (34), again suggesting a relationship between NaCh expression and uterine contraction. For example, the percentage of rat uterine smooth muscle cells that expressed Na⁺-based inward current increased from zero at day 5 of pregnancy to 90% by days 18 and 21 (34). These data, combined with the increase in mNa,2.3 mRNA just prior to delivery, and the rapid drop after, suggest that voltage-gated NaChs play either a direct or permissive role in uterine contractility.

The TTX sensitivity of Na⁺ currents in both human (35) and rat (34) uterine myocytes (1 µm required for complete block) is similar to that observed for the skeletal muscle NaCh isoform, raising the possibility that this isoform is expressed in uterus just prior to delivery. To determine whether previously cloned NaCh mRNAs increase in uterus as term approaches, Northern analysis was performed using uterine mRNA from days 15 and 19 of pregnancy and probes specific for the brain, heart, and skeletal muscle NaCh isoforms. None of these channels was detected in uterus at either time point (data not shown), suggesting that mNa,2.3 is the NaCh responsible for the Na⁺ current observed just prior to delivery.

**Relationship of mNa,2.3 to Other Sodium Channels**—As shown in Fig. 6, comparison of previously cloned full-length NaChs with mNa,2.3 grouped the mammalian and eel channels together in terms of similarity, suggesting they evolved from a common ancestor. We have designated this group as

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**Table 1**

| NaCh isoform | N | D1 | D2 | D2-3 | D3 | D3-4 | D4 | C |
|--------------|---|----|----|------|----|------|----|---|
| B-I          | 53| 49 | 39 | 56   | 32 | 55   | 50 | 56| 47|
| B-II         | 51| 49 | 37 | 55   | 36 | 56   | 50 | 57| 52|
| B-III        | 51| 48 | 33 | 55   | 32 | 54   | 50 | 57| 52|
| SKM1         | 47| 49 | 20 | 55   | 25 | 54   | 47 | 53| 45|
| H-1          | 43| 46 | 26 | 49   | 20 | 53   | 50 | 50| 44|
| Ee1          | 41| 47 | 20 | 50   | 26 | 53   | 41 | 50| 33|
| hNa,2.1      | 70| 72 | 60 | 65   | 70 | 84   | 74 | 80| 79|

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**Fig. 3. Northern blot analysis of the distribution of mNa,2.3 mRNA in AT-1 cells and mouse tissues.** Total RNA was isolated from cultured AT-1 cells and the indicated tissues from 4-week-old mice. 15 μg of total RNA was processed as described under "Experimental Procedures." Lane C: AT-1 cells; lane B: brain; lane H: heart; lane K: kidney; lane L: liver; lane S: skeletal muscle; lane U: uterus. The blot was exposed for 14 days.
vertebrate NaCh gene subfamily 1 (Na,1). The hNa,2.1, mNa,2.3, and rat glial NaCh are grouped in subfamily 2 (Na,2), most likely representing a second, evolutionarily distinct subfamily for vertebrates. The invertebrate channels from squid and Drosophila are most similar to each other, while the jellyfish channel stands alone as expected since the jellyfish represents the evolutionarily oldest animal with a nervous system.

In comparing ion channel amino acid sequence differences across species, it is often difficult to distinguish between isoform differences and simple species variation. The mNa,2.3 sequence is 68% identical to that of the human Na,2.1 clone, with the sequence differences evenly dispersed throughout the protein as opposed to being concentrated in nonmembranespanning regions, which are likely to be less important for channel function. Cross-species amino acid sequence identity between the same NaCh isoform is typically greater than 90%. For example, the skeletal muscle I (10, 11), brain type II (4, 6), and cardiac I (7, 8) NaChs share 92, 97, and 90% identity between human and rat species, respectively. Therefore, it is tempting to speculate that the mNa,2.3 clone represents a related but distinct NaCh isoform when compared with the hNa,2.1 NaCh. The difference in the number of unusually conserved positive charges in the second S4 region between the human and mouse clones does argue for two distinct isoforms. However, the similarity in the tissue specificity of transcript expression between human and mouse suggests that these two clones represent the same isoform from a functional standpoint.

While only the C-terminal quarter of the glial NaCh amino acid sequence has been reported (18), this sequence is 90% identical with the corresponding region of Na,2.3, raising the possibility that these two clones represent species homologs. In addition, the patterns of mRNA expression are similar. The glial channel was expressed in heart, and only low levels were observed in brain. Expression in uterus was not determined. However, as shown in Fig. 4, mNa,2.3 mRNA was high in mouse heart at PNI while the rat glial NaCh mRNA was low in rat heart at PNI relative to adult levels (18). Without the full-length rat glial sequence, it is not possible to determine whether the mNa,2.3 sequence reported here represents the same isoform as the rat glial protein, for C-terminal similarity may be misleading. The rat brain II and III NaChs represent different gene products, are 85% identical overall, but show 89% identity in repeat IV and the C terminus. It is premature to draw any conclusions with respect to the relatedness of the human Na,2.1, mouse Na,2.3, and the glial channel beyond the idea that they are clearly within the same NaCh gene subfamily.

CONCLUSION

The mNa,2.3 cDNA clone provides the first full-length amino acid sequence for a putative rodent NaCh that has unique features in regions known to be important in voltage-dependent activation and inactivation. mNa,2.3 is homologous to the human Na,2.1 clone and can be placed in the Na,2 NaCh gene subfamily. The conservation of usual sequence in functionally important domains between the hNa,2.1 and mNa,2.3 channels and the similar tissue-specific expression between human and mouse argue in favor of an important physiological role for members of this second mammalian NaCh gene subfamily. In addition, up-regulation of transcript levels in mouse uterus near delivery suggest an important role in control of uterine excitability. The mouse species will be valuable in determining...
the physiological role that these Na,2 subfamily NaChs play in tissue physiology. Unanswered questions concern the specific cells expressing the mNa,2.3 channel, its subunit composition, and the nature of the ionic current generated. Site-directed antibodies will be required to address the first two points while heterologous expression, if successful with the mNa,2.3 clone, will provide insight into the current phenotype. If additional subunits are required for expression, the AT-1 cells and native mouse tissue will provide the means by which to biochemically identify these proteins.

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