Analysis of Nedd8-Associated Polypeptides: A Model for Deciphering the Pathway for Ubiquitin-like Modifications

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ABSTRACT: Ubiquitin-like proteins modify target proteins, altering their activities or causing them to be slated for degradation. These modifications are used to efficiently regulate key events in the cell. To explore the set of proteins modified by a small ubiquitin-like protein, we have developed a proteomic approach. Affinity purification of an epitope-tagged Nedd8 allowed the identification of the majority of proteins known to be involved with the neddylation pathway. This purification not only isolated the known targets of neddylation but also the constellation of enzymes and complexes known to regulate neddylation and deneddylation, including the COP9 signalosome, Nub1, and enzymes in the neddylation cascade. This purification scheme can be applied to other small ubiquitin-like proteins, especially those with limited protein targets such as the SUMOs (1, 2, and 3), Isg15, or FAT10.

Small ubiquitin-like protein modifications are used to regulate a wide variety of events. When proteins are covalently modified, these peptides target them for destruction by the proteasome or cause changes to their target's activities. This system allows for rapid changes in the overall activity of the cell and, as such, is frequently used to regulate the cell cycle or respond to external stimuli (*1*, *2*).

The best known of these modifiers is ubiquitin. This 8.6-kDa protein modifies numerous other proteins in the cell. After ubiquitin is preprocessed by a C-terminal hydrolase, it is covalently attached to its targets by a three-protein relay of ubiquitylation enzymes (*3*). The first member of the relay is an activator protein, termed E1. The E1 adenylates the C terminus of ubiquitin and then transfers it to a cysteinyl side chain. It is then transferred to another cysteinyl group on the E2 or ubiquitin conjugator. The E3, or ubiquitin ligase, covalently attaches the ubiquitin to a lysine residue on its substrate with an isopeptide bond. Upon polyubiquitylation, proteins are targeted to the proteasome where they are degraded (reviewed in ref *4*). An example of this efficient mode of downregulation is the ubiquitylation and subsequent degradation of securin by the APC. Degradation of securin frees its binding partner separase, which can then trigger anaphase (reviewed in ref *5*). Some protein targets are mono-ubiquitylated and are not degraded. Histones are mono-ubiquitylated in transcriptionally active regions of DNA in higher eukaryotes. In yeast, histone H2B is mono-ubiquitylated on Lys123, resulting in transcriptional activation of several inducible genes (*6*−*9*).

Other ubiquitin-like modifiers include SUMOs, Nedd8, and FAT10. Using a similar three-protein cascade to modify their targets, these ubiquitin-like modifiers are used in a variety of regulatory roles. SUMO1 is well-known as a regulator of nuclear transport through its modification of RanGAP1, as well as a negative regulator of cytokine signal transduction through the modification of STAT1 in the JAK-STAT pathway (*10*, *11*). Nedd8 regulates the E3 ubiquitin ligase activity of the members of the cullin family (*12*, *13*). Other ubiquitin-like proteins, such as FAT10, have no known targets, and little is known about their biological functions (*14*).

Nedd8 was chosen as a candidate for this purification scheme because it is known to modify relatively few protein targets, and few of those that it does modify are slated for degradation. Nedd8 is an 81 amino acid, 6 kDa, ubiquitin-like protein. Similar to ubiquitin, it specifically covalently modifies proteins (*15*, *16*). Its only known targets are the cullins (*17*). As with ubiquitin, it has an E1 activator, the AppBP1/hUba3 heterodimer, an E2 conjugator, hUbc12, and an E3 ligase, tentatively identified as Rbx1, which, together, act as a three-stage relay to prepare Nedd8 and then covalently attach it to its target proteins (*16*, *18*, *19*). At this time, there are no other known E3s for Nedd8, although many cullin complexes do contain Rbx1.

Cullins are the only known targets of neddylation (*17*). The seven members of this family of proteins are generally components of SCF-like E3 ligase complexes and are involved in regulation of the cell cycle, cellular response to hypoxia, and DNA damage (*20*). Cullin complexes modified by Nedd8 show an increase in E3 ligase activity (*12*, *13*). When these responses are no longer needed, deneddylases can remove Nedd8 from its target proteins and shut off their E3 ligase activity (*21*, *22*).

Here, we report the isolation of Nedd8-associated proteins. The limited number of neddylated proteins provided us with

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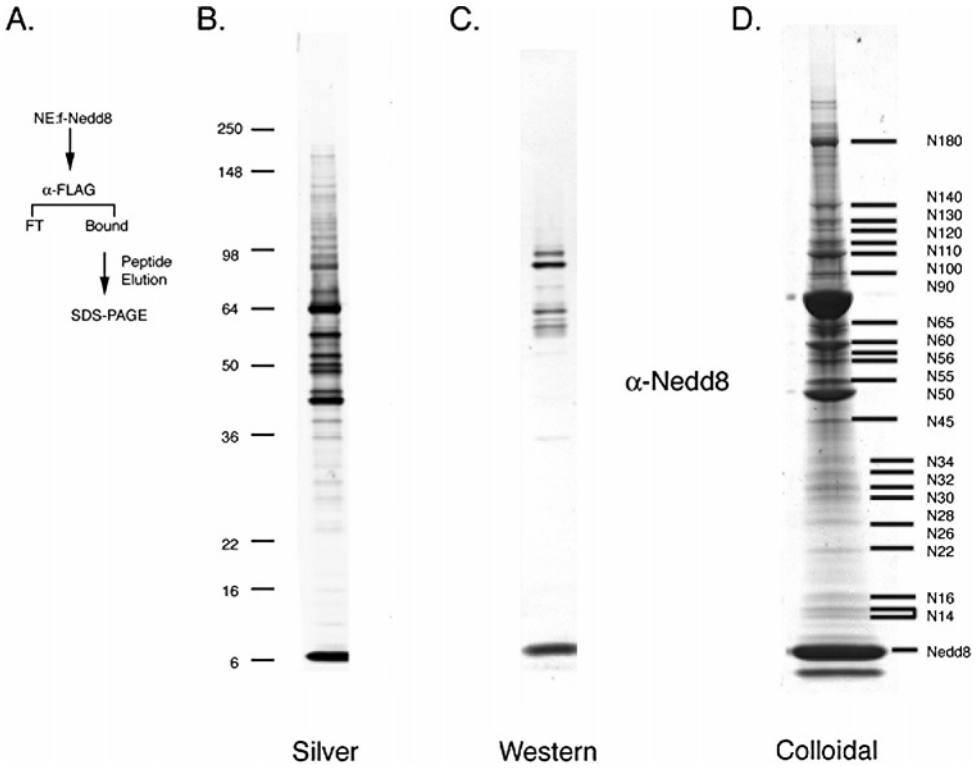

FIGURE 1: Purification of FLAG−Nedd8-associated proteins. (A) FLAG−Nedd8-associated proteins were purified from nuclear and cytoplasmic fractions by immunoprecipitation. (B) FLAG−Nedd8 293 nuclear extract was purified as indicated in A. A total of 0.5 g of nuclear extract was incubated with FLAG−resin and then washed with BC500 with 0.1% NP40. The bound proteins were FLAG-peptide-eluted and then separated on an SDS−polyacrylamide gel (4−20%), and proteins were visualized with silver stain. (C) Western blot with anti-Nedd8 antibodies of the FLAG-affinity-purified complex. (D) FLAG−Nedd8-associated proteins visualized with Colloidal Blue. Molecular masses of bands selected for ion-trap mass spectrometry (in kilodaltons) are indicated, and the proteins identified are indicated in Table 1.

an ideal system to test a proteomic approach as well as discover previously unknown targets of neddylation. Our purification scheme isolated essentially all known proteins associated with neddylation from the E1 conjugator to the deneddylating COP9 signalosome, indicating the utility of this technique for similar ubiquitin-like proteins.

RESULTS

Isolation of Nedd8-Containing Complex(es). To define the neddylation pathway and to identify novel substrates for neddylation, we isolated Nedd8-containing complexes. We created a stable 293-derived cell line expressing a FLAG epitope-tagged Nedd8. We subjected nuclear extract and S100 to affinity purification with M2 FLAG resin and eluted the bound proteins with FLAG peptide (Figure 1A). Samples were subjected to SDS−PAGE analysis, and the proteins were visualized using silver stain or Western blot analysis using antibodies against Nedd8 (parts B and C of Figure 1). Although the silver-stained gel showed a complex harvest of proteins, Western blot analysis of the affinity eluate revealed the presence of a few polypeptides representing neddylated species.

The affinity eluate was then concentrated by trichloroacetic acid precipitation, subjected to SDS−PAGE, and stained with Colloidal Blue staining (Figure 1D). The primary bands were then excised and identified by mass spectrometric sequencing. Multiple peptide sequences were determined in a single run by microcapillary reverse-phase chromatography (a custom New Objective 50-μm column terminating in a nanospray 15-μm tip), directly coupled to a Finnigan LCQ Deca quadrupole ion-trap mass spectrometer. This resulted in the identification of 38 proteins that are known to play a role in the Nedd8 pathway (Table 1).

Identification of Nedd8-Associated Components. Mass spectrophotometric analysis revealed four functionally distinct classes of proteins. Enzymes required for neddylation identified through mass spectroscopic analysis include UCH-L3, AppBP1, hUba3, and hUbc12 (Table 1). The second class of proteins, protein substrates for neddylation, was represented by Cul1, Cul2, Cul3, Cul4A, Cul4B, and Cul5. Other components of Cullin-containing complexes were also identified: elongin B/C, Skp1, MEI1, a SOCS-box protein, and Ddb1. A representative of the third class of proteins, deneddylases, was the COP9 signalosome, identified by six of its eight subunits. The fourth class of proteins found in this purification includes Nub1, an enzyme that recruits neddylated substrates to the proteasome for degradation. Finally, all but one subunit of the 20S proteasome was also associated with Nedd8. These results testify to the utility of our approach in delineating the up- and downstream components of the neddylation pathway.

Nedd8s Only Substrates Are the Cullins. To examine whether cullins are the only substrates for neddylation, the Nedd8-affinity eluate was subjected to gel filtration and the column fractions were subjected to Western blot analysis using Nedd8 antibodies (Figure 2A). This analysis revealed the presence of neddylated proteins exhibiting molecular weights consistent with that of the cullins peaking in fractions 28−30 of gel filtration (Figure 2B). Furthermore, analysis of the gel-filtration column fractions using antibodies to cullin

Table 1: Proteins Involved in Neddylation[a]

complex	components	biological function	relationship to Nedd8	sequence
Nedd8 E1	**AppBP1**	**E1 activator**	**activates Nedd8**	**N65**, **N90**
	hUba3			**N65**, **N56**
Nedd8 E2	**hUbc12**	**E2 cogugator**	**congugates Nedd8**	**N32**
UCH-L3	**UCH-L3**		**ubiquitin and Nedd8 C-terminal hydrolase**	**N30**
SCF	**Skp1**	**G1/S transition**	**Nedd8 modifies Cul1 activating**	**N22**
	Cul1		**E3 ligase activity**	**N110**, **N100**
	F box protein			
VEC	**Cul2**	**hypoxia**	**Nedd8 modifies Cul2 activating**	**N100**
	Rbx1		**E3 ligase activity**	**N16**, **N14**
	elongin B			**N14**
	elongin C			
	VHL			
	Cul5		**Nedd8 modifies Cul5 activating**	**N100**
	Rbx1		**E3 ligase activity**	**N16**, **N14**
	elongin B			**N14**
	elongin C			
	SOCS-box protein			
BCR3	**Cul3**	**cell division**	**Nedd8 modifies Cul3 activating**	**N110**
	Rbx1		**E3 ligase activity**	**N90**
	BTB domain proteins (MEI1)			
COP9 signalosome	**CSN1**	**cell cycle**	**deneddylates Cul1 and Cul2, inactivating SCF/VEC**	**N60**
	CSN2			**N55**
	CSN2			**N50**
	CSN3			**N45**
	CSN4			**N34**
	CSN5			**N26**
	CSN6			
	CSN7A/B			
	CSN8			
	zyxin		**reported to be associated with *C. elegans* COP9**	**N90**
DDB2	**Cul4A**	**NER**	**upon UV radiation, dissociates from CS,**	**N100**
	Ddb1		**Cul4A becomes neddylated and binds chromatin**	**N140**, **N130**
CSA	**Cul4A**	**NER**	**upon UV irradiation, binds RNA pol II**	**N100**
	others		recruits CSN and Cul4A is deneddylated	
	Cul4B			**N130**, **N120**, **N110**
	Nub1	**cell cycle**	**recruits neddylated proteins to proteasome**	**N90**
20S	α1	**targeted protein**	**may use the COP9 signalosome**	**N34**
	α2	**degradation**	**as an alternate lid**	**N28**
	α3			**N32**
	α4			**N32**
	α5			**N30**, **N32**
	α6			**N30**
	α7			**N32**
	α1			**N26**
	α2			**N26**
	α3			**N26**
	α4			**N28**
	α5			**N26**
	α6			**N32**
	α7			

[a] Proteins and protein complexes that are neddylated or involved in neddylation and deneddylation are listed here in four groups. Proteins isolated in our purification scheme are in bold.

1 or cullin 2 indicated a very similar pattern of immunoreactivity as that obtained with antibodies against Nedd8, attesting to the fact that they represent some of the neddylated proteins. When our results are taken together, they point to the cullins as being the only substrates for neddylation.

Cullin Complexes Do Not Directly Associate with the Proteasome. The association of the proteasome with neddylated components was intriguing. Notably, the COP9 signalosome resembles the 19S cap of the proteasome, and it has been postulated that the signalosome can replace the proteasome cap in some circumstances (*22*−*24*). COP9 has been reported to interact with the proteasome in *Arabidopsis*, but this interaction has not been verified in mammalian cells. It might be plausible that the cullin-containing complexes, which polyubiquitinate substrate proteins to prime them for degradation by the proteasome complex, might display a transient physical interaction with the proteasome subunits.

To assess this possibility, we examined the association of the proteasome with a Cul2-containing complex. To isolate a Cul2-containing complex, we developed an H1299-derived cell line stably expressing FLAG−VHL. The nuclear extract from these cells was purified by FLAG-affinity immunoprecipitation. The affinity eluate was fractionated by gel-filtration chromatography, and the column fractions were subjected to SDS−PAGE and subsequently analyzed by silver stain and Western blot analysis (parts A and B of

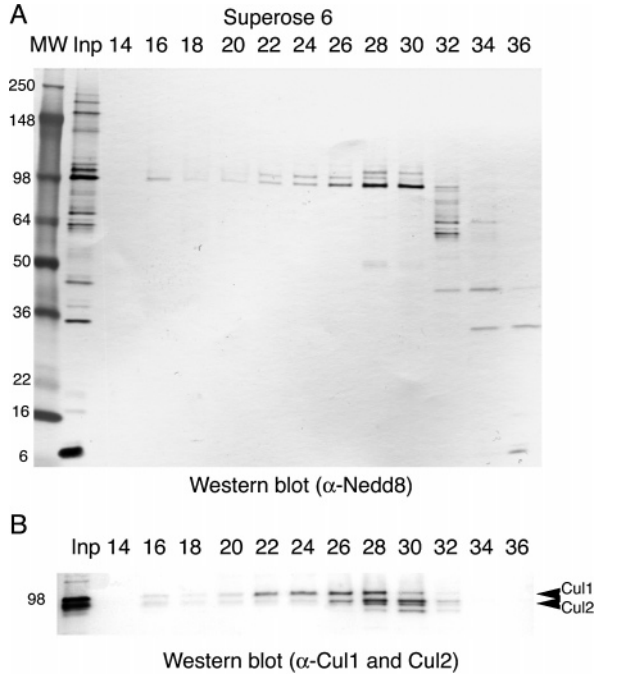

FIGURE 2: Cullins are the only substrates of Nedd8. FLAG−Nedd8-associated protein complexes were purified by immunoprecipitation and subsequent gel-filtration chromatography, subjected to SDS−PAGE, and visualized with Western blot analysis using α-Nedd8 antibodies (A) or α-Cul1 and α-Cul2 antibodies (B).

Figure 3). To identify the components of the VHL-containing complexes, the affinity eluate was also analyzed by mass spectrometric sequencing. This purification resulted in three distinct complexes (represented by Roman numerals in Figure 3). Significantly, none of the VHL-containing complexes yielded an association with the proteasome subunits. The largest complex was the TCP1 ring complex (TCiP), which chaperones VHL until it can be assembled in the VEC complex (*25*, *26*). The second complex is the VEC complex, containing members Cul2, VHL, elongin B/C, and Rbx1 (*27*, *28*). The smallest complex contains VHL and elongin B/C. These results indicate that the presence of the proteasome in Nedd8-affinity eluate represent the association of the proteasome with a different component of the pathway than that of cullin-associated E3 ligase complexes.

DISCUSSION

A single-step affinity purification of tagged Nedd8 reveals the array of protein complexes associated with this signaling pathway. These findings provide an example of a proteomic approach to the elucidation of protein modification networks and suggest how this method can be fruitfully applied to other protein modifiers in the ubiquitin family.

Our purification scheme delineated the entire network of Nedd8-associated proteins. This network may be divided into four classes of proteins. The first is the set of enzymes responsible for preparing Nedd8 to be covalently attached to its substrates. These are the C-terminal hydrolase, E1, E2, and E3. The second group consists of Nedd8 substrates, the cullin-containing E3 ligases. The third set includes the deneddylases that remove Nedd8 from its substrates. The fourth and final set of proteins identifies and targets neddylated proteins to the proteasome for degradation. Our purification yields representatives from all four groups (Table

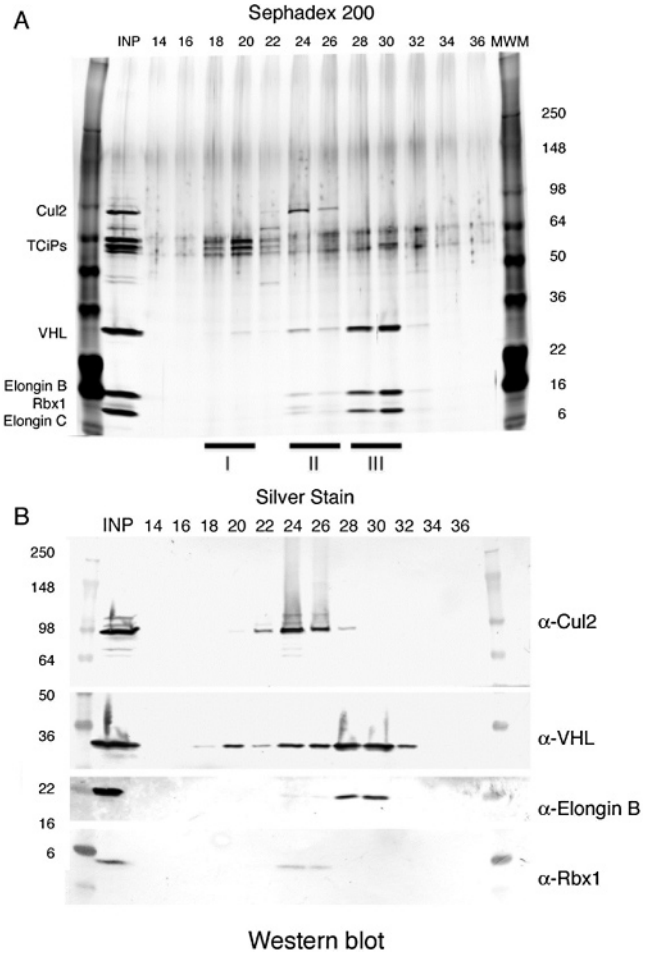

FIGURE 3: Copurification of COP9 signalosome with 20S proteasome is not due to association with cullin E3 ligase-associated complexes. FLAG−VHL-associated protein complexes were purified by immunoprecipitation and subsequent gel-filtration chromatography, subjected to SDS−PAGE, and visualized with silver stain (A) and Western blot (B) using the antibodies indicated at the right.

1). By choosing a well-known modifier, we have highlighted the efficiency of this technique.

Nedd8 modification has been extensively studied, and while new associations were unexpected, we did uncover some. The COP9 signalosome, a member of the third group of proteins associated with neddylation, deactivates cullin-containing complexes by deneddylating their component cullins and was represented by six of its eight subunits as well as zyxin, a protein whose worm homologue was previously known to associate with the *Caenorhabditis elegans* signalosome subunit CSN5 (*29*, *30*). Additionally and consistent with previous findings in *Arabidopsis* that COP9 interacts with the proteasome, our purification included all but one subunit of the 20S proteasome (*31*). Also of note in our purification was a TRIM11-like protein, LOC149603. This protein, like other TRIM family members, contains a RING-box motif, and future experiments will determine if it too has E3-like activity (*32*, *33*).

Hemelaar et al. and Gan-Erdene et al. used vinyl sulfone, a C-terminal electrophillic trap, to modify recombinant ubiquitin-like modifiers and thus identify their E1s, E2s, as well as enzymes involved in the removal of the ubiquitin-like modifier (*21*, *34*). Zhao et al. use HA-tagged SUMO1 to identify sumoylated substrates (*35*). Our technique,

immunoprecipitation of an epitope-tagged protein, is not limited to either a small set of enzymes or modified substrates and instead defines the entire network of proteins involved with the ubiquitin-like modifier. Moreover, our method allows us to perform our purifications in vivo in cell lines and not rely on recombinant protein added to in vitro cell extracts.

Small protein modifiers are ideal candidates for a proteomic approach to complex purification. They allow for the rapid identification of a subset of complexes in the cell. Because of the ease of the technique, cells may be subjected to different conditions and dynamic response of the cell may be observed. This information can be used to shed light on the network of pathways involved in stimulus response. These results demonstrate the efficiency of our approach in delineating the players in the neddylation pathway.

MATERIALS AND METHODS

Materials. Nedd8 and Cul2 antibodies were purchased from Zymed. Elongin B antibody was purchased from Santa Cruz. Elongin C (Anti SIII p15) antibody was purchased from Transduction Laboratories. Rbx1 (Roc1) antibody was purchased from USBiologicals. Anti-FLAG M2 antibody, antibody resin, pFLAG−CMV2, and FLAG peptide were obtained from Sigma.

Cell Lines. Nedd8 was cloned into pFLAG−CMV2 (Sigma). pFLAG−Nedd8 and a selectable marker for puromycin resistance were cotransfected into 293 human embryonic kidney cells with Fugene 6 (Roche). Transfected cells were grown in the presence of 2.5 μg/mL puromycin, and individual colonies were isolated, expanded, and analyzed by Western blot for FLAG−Nedd8 expression. A FLAG−VHL overexpressing H1299 cell line was created in a similar manner.

Purification of Nedd8-Associated Proteins. Nuclear and S100 cytoplasmic extracts were made using the Dignam et al. protocol (*36*). FLAG immunoprecipitation was performed with Anti-FLAG M2-agarose affinity gel (Sigma). After immunoprecipitation, this resin was washed with BC500 0.1% NP40 (20 mM Tris at pH 7.9, 500 mM KCl, 0.2 mM EDTA, 10% glycerol, 5 mM β-mercaptoethanol, and protease inhibitors). Five sequential elutions were performed with one resin volume each of 500 ng/μL FLAG peptide (Sigma). The resulting fractions were run on 4−20% gradient SDS−PAGE gels (Invitrogen) and subsequently Western blotted with relevant antibodies or silver-stained for total protein. Samples for sequencing were run on similar gels but were stained with Colloidal Blue (Invitrogen). This purification was compared to previous purifications to eliminate the background.

Size-Exclusion Chromatography. FLAG-immunoprecipitated proteins were run on a Superose 6 or Sephadex 200 gel-filtration column (Pharmacia). Fractions were subjected to SDS−PAGE and subsequently visualized with silver stain or Western blotted with appropriate antibodies.

Mass Spectrometric Peptide Sequencing. Protein bands of interest were manually excised from a 1D SDS−PAGE gel using a scalpel, destained using 200 μL of 200 mM ammonium bicarbonate in 50% acetonitrile for 30 min at 37 °C and then dried in a Speedvac. The bands were then subjected to 15 min of reduction/alkylation using 100 μL of 20 mM TCEP in 25 mM ammonium bicarbonate at pH 8.0 and 37 °C. The supernatant was then discarded, and the band was washed twice for 15 min with 200 μL of 25 mM ammonium bicarbonate. The band was then washed with 25 mM ammonium bicarbonate in 50% acetonitrile, dried in a Speedvac, and subsequently rehydrated overnight at 37 °C with 20 μL of 0.02 μg/μL modified trypsin (Promega) in 40 mM ammonium bicarbonate. The supernatant was then removed to a clean tube, while the band was further washed for 30 min at 37 °C with 40 mM ammonium bicarbonate. The supernatants were then combined with 4 μL of glacial acetic acid.

Multiple peptide sequences were determined in a single run by microcapillary reverse-phase chromatography (a self-packed New Objective 75-μm column terminating in a nanospray 15-μm tip), directly coupled to a ThermoElectron DecaXPplus quadrupole ion-trap mass spectrometer. The data were acquired using either a top three method or a top six method to obtain MS and MS/MS data. The resulting masses and MS/MS spectra were searched against the nonredundant NCBI database using TurboSEQUEST Browser (*37*).

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