Rat Coagulating Gland Secretion Contains a Kinesin Heavy Chain-like Protein Acting as a Type IV Transglutaminase Substrate †

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Rat Coagulating Gland Secretion Contains a Kinesin Heavy Chain-like Protein Acting as a Type IV Transglutaminase Substrate†

Carla Esposito,*,‡ Loredana Mariniello,§ Anna Cozzolino,§ Angela Amoresano,|| Stefania Orrù,‡ and Raffaele Porta§

Department of Chemistry, University of Salerno, 84081 Baronissi (SA), Italy, and Department of Food Science and International Mass Spectrometry Facilities Centre, CNR-University of Naples, Naples, Italy

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ABSTRACT: By a proteomic approach, we demonstrated in rat coagulating gland secretion the presence of a 120 kDa protein which shares at least 80% identity at the amino acid level with the most closely related kinesin heavy chain codified by the kinesin superfamily protein *Kif5c* gene. In addition, we identified 30 and 66 kDa proteolytic fragments of such a kinesin heavy chain-like protein, corresponding to the 73−299 N-terminal and 300−860 C-terminal regions, respectively. Finally, we demonstrated the occurrence in coagulating gland secretion of a 200 kDa protein probably derived by cross-linking reaction of the kinesin heavy chain-like protein with type IV transglutaminase. In fact, kinesin heavy chain-like protein and its 66 kDa proteolytic fragment were also found to act as effective acyl donor substrates for the enzyme in vitro.

Transglutaminases (TGases,[1] EC 2.3.2.13) are Ca^{2+}-dependent enzymes that catalyze the formation of covalent isopeptide bonds between the γ-carboxamide group of protein-bound glutaminyl residues and either the ϵ-amino group of lysyl residues, present in the same or in a different polypeptide chain, or primary amines (*1*−*3*). Several distinct TGase molecular forms have been described, and their respective cDNAs and deduced primary protein sequences have been resolved (*3*). These include plasma factor XIIIa, keratinocyte TGase, epidermal TGase, tissue TGase (type II), and prostate TGase (type IV). Rat type IV TGase is a secretory glycoprotein that is responsible for the rapid cross-linking of the copulatory plug after mating (*4*). Moreover, the enzyme produces polymeric forms of one of the major proteins secreted from rat seminal vesicles, which acquires the capability of both binding to the epididymal sperm cells and suppressing their immunogenicity (*5*−*7*). The latter role of type IV TGase has been hypothesized also in humans since TGase activity has been revealed both free in the human seminal plasma and bound on the spermatozoon surface (*8*). The structure and the properties of the rat type IV TGase have been investigated in detail (*9*−*11*). Spectrometric analyses of purified coagulating gland secretion (CGS) TGase have revealed the high degree of complexity of such enzyme. The N-terminal end is blocked, and residues Asn408 and Asn488 are glycosylated with both high-mannose and complex-type glycans. Moreover, mass spectral analysis demonstrated the presence of a lipid anchor retained during the enzyme secretion process (*9*). The molecular mechanism by which TGase secretion is achieved remains to be elucidated, since TGase does not show any known sequence which could lead it to the secretory machinery (*12*). Several reports indicate that type IV TGase is secreted via an apocrine fashion (*13*, *14*). This mechanism, which can only occur in polarized cells, seems linked to sex-related glands (*15*) and does not involve the endoplasmatic reticulum or the Golgi complex being the protein secreted through the formation of blebs into the plasma membrane.

Recently, we have found that a GTP hydrolyzing activity with a high affinity for the susbtrate is present in CGS (*16*). The GTP hydrolyzing activity was demonstrated to be linked to a high molecular mass protein(s) with an electrophoretic mobility higher than 100 kDa, and a low molecular mass enzyme(s) of about 30 kDa. Since GTP acts as a negative modulator for type IV TGase (*16*), a key role of the nucleotide related to TGase and GTPase activities could be hypothesized. It has long been shown that GTP is necessary for cell secretion (*17*, *18*), and today small G-proteins are going to be demonstrated as increasingly involved in protein import−export (*19*, *20*). Therefore, it is reasonable to suppose that at least the 30 kDa GTPase activity plays a role in the intracellular transport and apocrine secretion of rat type IV TGase.

In this paper we report data showing that a kinesin heavy chain (KHC)-like protein occurs in CGS and that such KHC-like protein and its 66 kDa C-terminal proteolytic fragment are able to act as type IV TGase substrates.

The results were discussed in relation to the previously detected occurrence of GTPase activity in CGS, and to the

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* Address correspondence to this author at the Department of Chemistry, University of Salerno, 84081 Baronissi (SA), Italy. Tel: +39089965298, Fax: +39089965296, E-mail: esposito@chem.unisa.it.

‡ Department of Chemistry, University of Salerno.

§ Department of Food Science, CNR-University of Naples.

|| International Mass Spectrometry Facilities Centre, CNR-University of Naples.

[1] Abbreviations: TGase, transglutaminase; CGS, coagulating gland secretion; MALDIMS, matrix-assisted laser desorption ionization mass spectrometry; KHC, kinesin heavy chain; KIFs, kinesin superfamily proteins; Spd, spermidine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis.

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still unexplained molecular mechanism of the type IV TGase secretory process.

MATERIALS AND METHODS

Materials. [^{14}C]Spermidine (Spd) trihydrochloride (specific activity 110 mCi/mmol) was obtained from Amersham (Buckinghamshire, U.K.). Trypsin, dithiothreitol (DTT), iodoacetamide, Spd, glycerol, and α-cyano-4-hydroxycinnamic acid were purchased from Sigma. Econo-Pac Serum IgG purification Kit, Prestained Precision Protein Standards, and reagents for electrophoresis and immunoblot analysis were from Bio-Rad. Polyclonal antibodies against rat type IV TGase were raised in our laboratory (*9*). All other reagents and solvents were of the highest purity available from Carlo Erba (Milan, Italy).

Extraction of Coagulating Gland Secretion. Sexually mature male Wistar rats (250−300 g), lightly anesthetized with diethyl ether, were killed by cervical dislocation. The coagulating glands were dissected, and the secretions were removed by gentle pressure. The obtained secretion, containing between 150 and 300 mg of protein/mL, was diluted (1:1) with glycerol and without centrifugation was stored at −20 °C until use.

SDS−PAGE. SDS gel electrophoresis were performed on 7.5% linear slab gels. Molecular masses of protein bands were estimated by comparing their electrophoretic mobility to those of Bio-Rad Prestained Precision Protein Standards (10, 15, 25, 37, 50, 75, 100, 150, and 250 kDa).

In-Gel Digestion of Proteins. Coomassie blue-stained protein-containing bands were excised from the gel and washed first with acetonitrile and then with 0.1 M ammonium bicarbonate. Protein samples were reduced by incubation in 10 mM DTT for 45 min at 56 °C and alkylated with 55 mM iodoacetamide in 0.1 M ammonium bicarbonate for 30 min at room temperature in the dark as previously described (*21*). The gel particles were then washed with ammonium bicarbonate and acetonitrile. Enzymatic digestions were carried out with trypsin (15 ng/mL) in 50 mM ammonium bicarbonate, pH 8.5, at 4 °C for 4 h. The buffer solution was then removed, and a new aliquot of the enzyme/buffer solution was added for 18 h at 37 °C. A minimum reaction volume, sufficient for complete rehydration of the gel, was used. Peptides were then extracted by washing the gel particles with 20 mM ammonium bicarbonate and 0.1% trifluoroacetic acid in 50% acetonitrile at room temperature and then lyophilized.

MALDIMS Peptide Mapping. MALDI mass spectra were recorded using a PerSeptive Biosystem Voyager DE Instrument. A mixture of analyte solution and α-cyano-4-hydroxycinnamic acid [10 mg/mL in acetonitrile/ethyl alcohol/0.1% trifluoroacetic acid (1:1:1, v/v/v)] was applied to the sample plate and dried under vacum. Mass calibration was performed with insulin at 5734.5 Da and a matrix peak at 379.3 Da as internal standards. Raw data were analyzed by using computer software provided by the manifacturers and reported as average masses.

Database Searching with MALDI Data. The set of peptides obtained from the different protein bands was inserted into several search programs, available on the web, to search the nonredundant protein sequence database (NCBInr), taking advantage of the specificity of the proteolytic enzyme used for the hydrolysis and the taxonomic category of the sample. Search programs compare the set of experimentally found masses to virtual enzymatic digestions for each protein in the explored database and then generate a ranked output of the most likely candidates. A score (probability) of 1.0, in a 0.0−1.0 range, means that experimental mass signals match exactly predicted peptides within a specific protein in the database (*22*). In this work, cysteine was set as *S*-carbamidomethylcysteine, whereas methionine was considered both unmodified and oxidized; up to four incomplete cleavages per peptide were considered. Only experimental values within 0.05% accuracy were recorded. Different protein mass ranges were selected for each analyzed protein band. Three independent sets of experiments were performed.

TGase Assay. Enzyme activity was assayed by a radiometric method (*9*) based on the Ca^{2+}-dependent incorporation of isotopically labeled Spd into CGS proteins. The assay mixtures (50 μL), containing 125 mM Tris-HCl buffer, pH 8.0, 10 mM DTT, 40 μM [^{14}C]Spd, and 40 μg of CGS proteins, were incubated at 37 °C for 2 h in the presence of 1.5 mM SDS in order to activate the enzyme (*9, 10*). Blanks were simultaneously run with radioactive Spd in the presence of 5 mM EGTA. The reactions were stopped by EGTA addition (final concentration, 10 mM). The separation of the free amine from the labeled protein and the visualization of the latter were achieved by SDS−7.5% PAGE and fluorography (*23*). To evaluate the ability of the CGS proteins to form cross-linked heteropolymers containing prostate TGase, we incubated the crude prostatic fluid in the absence of Spd by using a assay mixture composition the same as that described before. The separation and the visualization of the protein-modified forms were performed by SDS−7.5% PAGE and Western blotting analyses.

Immunoblot Analyses. Primary antibody to CGS TGase obtained as previously described (*9*) was affinity-purified from rabbit polyclonal antisera following the manufacturer's protocol (Econo-Pac System). The proteins were transferred electrophoretically onto nitrocellulose strips. Binding of primary affinity-purified antibody, directed against purified CGS TGase, was revealed using horseradish peroxidase-conjugated goat anti-rabbit IgG and 4-chloro-1-naphthol/hydrogen peroxide as chromogenic agents.

RESULTS

Rat CGS Protein Mass Spectrometric Analysis. Mass spectrometric analyses were performed on the Coomassie blue-stained CGS proteins excised from SDS−polyacrylamide gels. The 7.5% SDS−PAGE pattern (Figure 1, lane 1) shows predominant molecular species having high molecular mass. To improve the separation of higher molecular mass proteins, the electrophoretic analysis was performed until the 50 kDa standard reached the bottom of the gel. In this way, proteins ranging from 300 to 66 kDa were well separated (Figure 1, lane 2). Each band was reduced, carboxymethylated, and digested with trypsin as described under Materials and Methods, and the peptide fragment mixtures were then directly analyzed by MALDIMS. The NCBInr search of the series of tryptic peptide masses obtained from 120, 70, and 30 kDa protein spectrometric analyses allowed their identification in the protein database.

It is worth noting that in-gel enzymatic digestions constitute a very complex mixture. In fact, the occurrence of

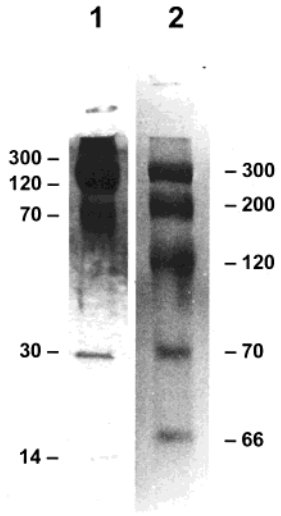

FIGURE 1: SDS−PAGE pattern of CGS proteins. Ten micrograms of CGS proteins was subjected to SDS−7.5% PAGE and run at different times (lane 1, 90 min; lane 2, 120 min). The proteins were revealed by Coomassie staining. The positions and corresponding molecular weights of CGS proteins are indicated.

peptide fragments containing missed cleavage sites and/or posttranslational and chemical modifications allows two or more peptides of the mixture to exhibit the same molecular mass. This phenomenon is more frequent as the molecular mass of the investigated protein increases. Nevertheless, the protein identification is certain and unambiguous by considering only mass values with 0.05% mass accuracy originated by three independent sets of experiments.

Table 1 shows that the 120 kDa protein was identified as the mouse neuronal kinesin heavy chain (KHC) (NCBInr accession number: P28738), a member of the kinesin superfamily proteins (KIFs), codified by the *Kif5c* gene. MALDIMS analysis allowed us to verify that 80% of the 120 kDa protein was identical to the entire neuronal KHC sequence (Figure 2); the protein was listed in the ranked output of several search programs in the first position with a very high score compared to the other candidates (probability = 0.98). In addition, the set of peptides obtained from the tryptic digest of the 70 kDa protein band, reported in Table 2, matched exactly the sequence of rat dorsal prostate TGase, with a probability of 1.0, thus confirming our previous results obtained by using different techniques (*9*). Finally, NCBInr search of the series of peptide masses obtained from spectrometric analysis of the 30 kDa protein band identified two molecular species, the first of which was assigned to the enzyme carbonic anhydrase (EC 4.2.1.1) with a probability of 1.0. The second molecular species identified in the 30 kDa protein band seems to correspond to the N-terminal fragment of mouse neuronal KHC. In fact, it shares about 45% identity with the N-terminal 340 amino acid long motor region of KHC (Figure 2, black boxes). Also, the peptide masses obtained by analyzing the 66 kDa protein are relative to 120 kDa protein fragments (Table 4), in particular to the KHC C-terminal region from residue 300 to residue 860, although only 17.5% coverage was obtained (Figure 2).

The mass spectral analysis of the CGS protein having an electrophoretic mobility of about 200 kDa assigned signals occurring in both rat dorsal prostate TGase and neuronal KHC sequences (Table 3). It should be underlined that only a portion of TGase was detected as indicated by the presence of a set of peptides identifying the TGase region from residues 275 to 588, whereas neuronal KHC was present from the N-terminal end to almost all the protein (855/1027). Similar results were obtained when the protein band of about 300 kDa was analyzed, even if weak spectra of it were detected (data not shown).

Table 1: NCBInr-Search of Incognite Protein at 120 kDa[a]

MH^+	KHC sequence	MH^+	KHC sequence
3752.7	284−315	1824.2	189−204
2988.9	545−571	1806.1	828−842
2841.6	461−485		874−890[c]
2832.7	300−323[b]	1798.5	145−160
2782.2	977−1002	1718.8	671−685
2705.6	547−571	1669.8	1−14[b]
2680.2	215−238	1491.0	215−227
2665.6	228−253		275−286
	828−849	1475.6	239−253
2641.7	523−545		287−299[b]
2636.7	192−214	1434.9	799−810
2618.1	447−468	1425.9	607−617[b]
2589.1	112−132	1390.3	33−45
	733−753		100−111
2565.1	424−444		689−697
2546.3	618−639[b]	1359.3	778−789
2537.1	790−810		358−369
2475.9	445−465	1312.6	763−772
2409.2	830−849	1296.9	152−162
	725−744	1272.9	546−556
2402.9	52−72[b]	1250.6	415−423
2385.9	168−188[b]	1233.9	287−297[b]
	258−280	1214.2	1017−1027
2312.9	790−808	1204.1	719−732
2228.6	607−624[b]	1200.9	846−855
2154.9	298−315[b]		864−873
2143.9	15−32	1179.6	423−431
2108.6	73−92		893−901
	782−799	1123.2	790−798
2099.5	254−274		881−890[c]
2087.6	811−829	1117.9	594−603
2058.7	809−827		708−716
2049.7	654−670	1094.2	415−422
2036.3	223−241		892−899
2001.1	697−712	1049.1	754−762
1989.6	738−753	1004.2	752−759
1885.1	152−167		719−726
	486−501	954.1	346−352
1875.7	589−604	938.9	316−323
1858.9	572−588		
	850−864[b]		

[a] Protein mass range: 70−120 kDa. [b] Peptides containing *S*-carbamidomethylcysteine. [c] Peptides containing oxidized methionine.

Rat CGS Protein Immunoblotting Analysis. To confirm that the 200 kDa protein band contains rat type IV TGase, immunoblotting analyses of CGS proteins were performed by using affinity-purified anti-TGase antibodies. Lane 1 of the pattern reported in Figure 3A shows a main band of 70 kDa corresponding to the enzyme and a weak signal relative to the protein of 200 kDa. Moreover, a signal relative to a band at the top of the 7.5% separating gel and another signal relative to high molecular weight polymers, which do not enter the 4% stacking gel, were also recognized by the antibodies used whereas the molecular species having electrophoretic mobility of 30 kDa was not recognized (data not shown). As shown in lane 2 of Figure 3A, 2 h incubation of crude CGS proteins, under optimal TGase experimental conditions, produced a marked increase of the signal of the

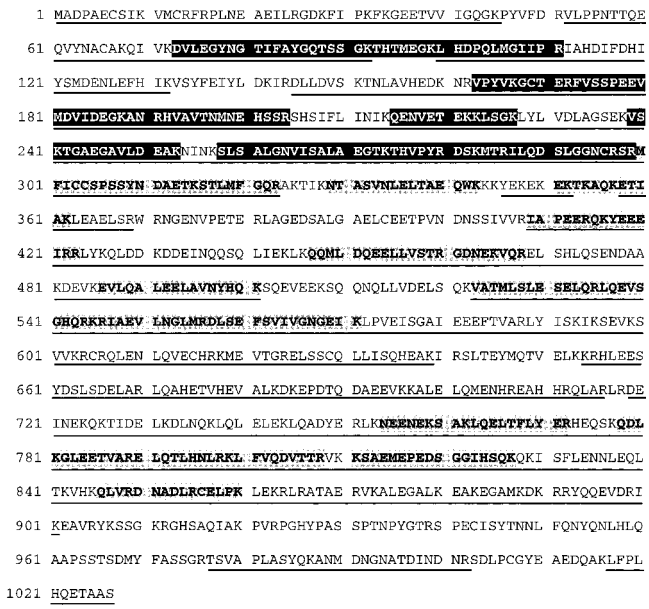

FIGURE 2: Amino acid sequence of mouse neuronal KHC. Sequences underlined represent 120 kDa protein fragments detected by MS spectral analyses. Black and gray boxes indicate regions that KHC shares with 30 and 66 kDa CGS proteins, respectively. The data are the results of three independent experiments.

Table 2: NCBInr-Search of Incognite Protein at 70 kDa[a]

MH$^+$	TGase sequence	MH$^+$	TGase sequence
3242.9	5−32	1853.3	17−32
3213.9	617−644[b]	1720.1	654−668
3124.1	323−349	1659.9	163−175
3005.1	174−201	1522.5	288−300
2990.3	248−274[b]	1477.4	381−391
2967.1	275−300	1465.3	275−287
	324−349	1444.1	442−454
2922.5	455−480	1429.2	33−44
2795.1	455−479	1406.1	546−558
2640.1	512−534	1366.1	657−668
	582−604[b]	1223.9	594−604
2544.1	594−616	1160.5	640−649
2313.1	288−307	1145.3	82−91
2202.9	419−436	1101.1	446−454
2174.6	420−437	1068.1	654−662
2109.1	308−323	996.7	350−359
2046.5	420−436	949.2	37−44
1982.9	92−111		437−445
1858.9	413−427		

[a] Protein mass range: 50−100 kDa. [b] Peptides containing *S*-carbamidomethylcysteine.

Table 3: NCBInr-Search of Incognite Protein at 66 kDa[a]

MH$^+$	KHC sequence	MH$^+$	KHC sequence
2989.3	545−571	1884.7	486−501
2830.5	300−323[b]	1829.0	811−827
2639.1	523−545	1375.1	351−362
2616.4	447−468	764.0	349−354
2313.9	790−808		754−759
1947.6	409−423		

[a] Protein mass range: 60−120 kDa. [b] Peptides containing *S*-carbamidomethylcysteine.

200 kDa protein band with a concomitant decrease of the intensity of the TGase band. These results suggest that cross-linkings occur between the 120 kDa protein and the enzyme itself. The pattern obtained when the incubation was performed in the presence of EGTA (lane 1), that caused the complete loss in the enzyme activity (*3*, *9*), was identical to that observed when CGS proteins were not incubated.

Table 4: NCBInr-Search of Incognite Protein at 30 kDa[a]

MH$^+$	carbonic anhydrase sequence	MH$^+$	KHC sequence
3529.9	57−88	2973.5	163−188[b]
2917.8	226−250	2385.6	168−188[b]
2659.7	132−157		258−280
2641.2	89−111	2175.4	93−111
2584.2	57−79	2109.1	73−92
2512.6	89−110	1824.8	189−204
2070.1	9−26	1490.9	215−227
2052.9	1−17		275−286
1992.9	148−166	1476.4	239−253
1839.6	111−125		287−299[b]
1776.8	212−226		
1711.1	112−125		
1679.2	132−147		
1582.8	113−125		
1241.5	158−168		
999.2	148−157		
965.4	80−88		
687.1	126−131		

[a] Protein mass range: 10−120 kDa. [b] Peptides containing *S*-carbamidomethylcysteine.

Table 5: NCBInr-Search of Incognite Protein at 200 kDa[a]

MH$^+$	KHC sequence	MH$^+$	TGase sequence
2727.9	168−191[b]	2312.8	288−307
2639.0	523−545	1847.0	275−290
2546.1	618−639[b]	1465.1	275−287
2405.9	763−781	1406.3	546−588
2385.4	168−188	1250.1	535−545
	258−280		
2155.1	298−315[b]		
2124.0	173−191		
2108.2	73−92		
	782−799		
1969.3	773−789		
1712.9	640−653		
1704.0	719−732		
	329−343		
1657.4	15−28		
1594.3	224−238		
1475.6	239−253		
	287−299[b]		
1427.1	607−617[b]		
1312.6	763−772		
1233.9	287−297[b]		
1200.9	846−855		
1123.0	1−10		

[a] Protein mass range: 70−250 kDa. [b] Peptides containing *S*-carbamidomethylcysteine.

Acyl Donor TGase Substrate Identification in Rat CGS. The possibility that the 120 kDa protein acts as an acyl donor substrate of TGase was explored by incubating CGS in the presence of [^{14}C]Spd, an effective acyl acceptor of the enzyme, and by detecting radioactivity incorporation. Labeled proteins were revealed by 7.5% SDS−PAGE of the incubation mixture and fluorography of the gel. Lane 5 of Figure 3B, corresponding to CGS proteins incubated in the presence of [^{14}C]Spd, shows a marked incorporation of radioactivity not only into the 120 kDa protein but also into the 200 and 300 kDa proteins occurring in CGS. Evident radioactive signals were also detectable at the level of the TGase band and, more faintly, at the level of the 66 kDa protein. Signals of radioactive polymers, which do not enter the 4% stacking

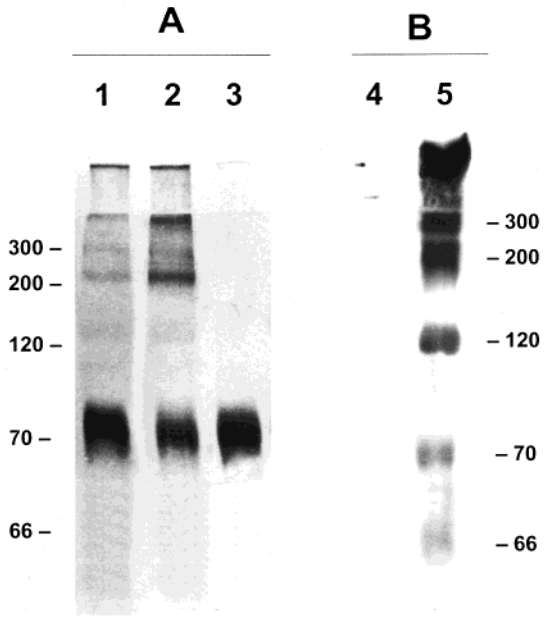

FIGURE 3: Western blot (panel A) and fluorography (panel B) analyses. Aliquots of 50 μL mixture assays, containing 10 μg of CGS proteins, incubated for 2 h at 37 °C in the presence (lane 1) or absence (lane 2) of 5 mM EGTA, were analyzed by Western blot (panel A). 0.5 μg of purified enzyme (lane 3) was used as control. Aliquots of 50 μL mixture assays, containing 10 μg of CGS proteins, incubated with [^{14}C]Spd in the presence (lane 4) or absence (lane 5) of 5 mM EGTA were analyzed by fluorography of the gel. The positions and corresponding molecular weights of CGS proteins are indicated.

gel, were also detected. Labeling of CGS proteins was completely inhibited when EGTA was present in the reaction mixture (Figure 3B, lane 4).

DISCUSSION

Rat CG is an androgen-dependent tissue synthesizing few major secretory proteins (*24*). Besides the well-known type IV TGase (*4*, *6*), we have recently found in CGS a GTP hydrolyzing activity associated with different molecular species of both high and low molecular weight (*16*). The proteomic approach, utilized in this work, provided for the first time evidence of the presence of a 120 kDa KHC-like protein in CGS. In more detail, the detected protein was found to share at least 80% identity with the amino acid sequence of mouse neuronal KHC (Figure 2). It is well-known that kinesins constitute a large superfamily of proteins (KIFs) of microtubule-dependent ATPase motors that play important roles in vesicle or organelle transport and cell division (*25*). The kinesin molecule consists of two 117 kDa KHCs and two 64 kDa kinesin light chains (*25*). A large number of different kinesins has been detected so far by molecular cloning, and three major types of KIFs have been identified according to the position of the motor domain. KHC itself forms a family, and mammals appear to have two or more genes encoding KHC and *kif5c* is a member of this family. *Kif5c* has been identified in mouse, and it is specific to nerve tissue (*26*−*28*), even though comparative analysis between 116 kDa KHC from human prostatic tumor cells and bovine brain showed that the two polypeptides were indistinguishable (*29*). KHCs share a conserved N-terminal motor region of approximately 340 amino acids, which generates force and binds ATP and microtubules (*25*). It is interesting to note that all the members of the three superfamilies of motor proteins (ATPase kinesins and myosins and GTPase dyneins) share a similar motor domain (30−50% amino acid identity) that can function autonomously as a force-generating element (*30*).

Mass spectrometric experiments allowed us to identify also the two molecular species responsible for the band having an electrophoretic mobility at about 30 kDa. The first protein represents carbonic anhydrase, the presence of which in CGS was already detected with a different technique (*31*). The second molecular species corresponds to the N-terminal region of KHC, since the recorded peptide signals covered 45% of its 1−340 amino acid sequence. The simplest interpretation of this result is that this 30 kDa protein is a proteolytic fragment of the 120 kDa KHC-like protein containing the entire nucleotide-binding domain. The observation that peptide signals of the C-terminal region of the KHC-like protein occur in the 66 kDa protein band of the CGS electrophoretic profile confirms such a hypothesis and is consistent with previous findings showing that in vitro proteolysis of KHC generates a 45 kDa fragment endowed with ATPase activity (*32*). Our results allow the interesting speculation that the 120 kDa KHC-like protein and its 30 kDa N-terminal proteolytic fragment, respectively, could be responsible for the high and low molecular weight GTPase activities previously observed in CGS (*16*). Since classical kinesins recognize ATP instead of GTP, it could be reasonable that some structural (posttranslational?) differences among KHC and the CGS 120 kDa protein exist at the level of the nucleotide-binding pocket. The concomitant presence of both type IV TGase and GTPase activities in CGS leads to the intriguing parallel with the type II TGase/GTP-binding protein. The hydrolysis of such an enzyme occurring in HeLa cell cytosol and rabbit liver nuclei produces a fragment of 36 kDa containing the GTP-binding domain (*33*, *34*) which copurifies with the entire protein throughout the purification protocol (*33*). We previously reported that the CGS 30 kDa fragment copurifies with type IV TGase because of the strong interactions existing between the two molecules which are separable only utilizing GTP affinity chromatography (*16*). Therefore, the association of TGase and 30 kDa GTPase activities in CGS seems to mimic the situation occurring in other biological districts where type II TGase is present. In fact, type II TGase is a dual enzymatic protein possessing also GTPase activity, and, thus, able to play two biological functions (*35*), the activation/deactivation of one of which may be under the control of multiple factors (*36*). In conclusion, we speculate that different regulatory signals could modulate the activity and function of the 30 kDa GTPase/type IV TGase complex in CGS.

Another important aspect concerns the mechanism by which type IV TGase is secreted by CG. Several reports indicate that rat prostate TGase is secreted via an apocrine fashion (*13*, *14*), even though this mechanism, up to now, is not fully clarified. In fact, both the energy dependence of the apocrine secretion and also the existence of a protein acting as delivery system have not been yet demonstrated. It was also not explained how apocrine proteins are selected for the transport into the blebs. It is reasonable to presume that ancillary proteins ensure the formation of blebs into the plasma membrane. This hypothesis is supported by our recent

findings obtained with the recombinant enzyme (unpblished experiments). In fact, we have synthesized the full-length cDNA and expressed the recombinant enzyme in MDCK cells. Immunological studies have demonstrated that the recombinant enzyme was not revealed in culture media of MDCK cells, although these cells possess an apocrine pathway when polarized during cell culture conditions (*37*). Therefore, the correct extrusion mechanism is probably achieved only in CG cells where the KHC-like and/or its proteolytic fragment could act as ancillary protein(s) necessary for the secretion of the enzyme. Since it is well-known that small G proteins regulate protein import−export (*19*, *20*), we speculate that CGS proteins endowed with GTPase activity bind TGase to address the enzyme to the extracellular environment. This hypothesis is supported by recent three-dimensional structure and kinetic studies that have uncovered intriguing parallels between motor proteins and G proteins, thus suggesting functional overlaps between G proteins and motors (*38*). For example, as the GTPase dynamin may generate mechanical activity during endocytosis, so homologue motor proteins may function as switches or mechanical gates rather than generating movement (*29*). This perspective is supported by the evidence that motor proteins and GTPases seem to use similar initial structural movements in the nucleotide-binding domain to produce distinct reactions in other regions of the molecule (*38*).

We have finally demonstrated the occurrence in the crude CGS of high molecular weight heterologous complexes containing cross-linked KHC-like protein and TGase. Moreover, both the 120 kDa KHC-like protein and its 66 kDa proteolytic fragment were shown to act as substrates for type IV TGase. These results are correlated to the finding that kinesin and myosin derive from a common ancestor gene (*39*). In fact, it is well-known that myosin and also other cytoskeletal proteins are type II TGase substrates (*40*). The physiological significance of this event has been related to the stabilization of the cytoskeleton as well as of the extracellular matrix (*41*). In CGS, the reason why KHC-like protein acts as a type IV TGase substrate remains to be elucidated.

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