Jaw1 is a lymphoid-restricted protein localized to the cytoplasmic face of the endoplasmic reticulum (ER) and is a member of a recently recognized class of integral membrane proteins that contain carboxyl-terminal membrane anchors. The carboxyl-terminal 71 amino acids of the Jaw1 protein, which contain a hydrophobic membrane spanning region, are sufficient to target a heterologous protein to the ER. By discontinuous sucrose gradient ultracentrifugation, differential sedimentation was noted for the four major Jaw1 protein isoforms, with two of the forms predominantly soluble and two microsome-bound. Pulse-chase immunoprecipitations suggest a post-translational modification of two major isoforms of the protein resulting in an increase in mobility on SDS-polyacrylamide gel electrophoresis. In vitro translation studies are compatible with a post-translational processing event that results in cleavage of a short 36 amino acid luminal domain. These findings define a carboxyl-terminal domain of the Jaw1 protein that is both necessary and sufficient for ER localization. In addition, the processing of the small luminal domain of Jaw1 represents a novel post-translational protein modification performed by the endoplasmic reticulum.

The insertion of type I membrane proteins ($\text{N}_{\text{exo}}\text{C}_{\text{cyt}}$) and all secreted proteins into the endoplasmic reticulum generally occurs co-translationally and requires the participation of signal recognition particle (SRP) ($\text{R}$) (1, 2). The 54-kDa subunit of SRP binds to the amino-terminal signal sequence of nascent protein chains and delivers the nascent protein-ribosome complex to the ER membrane where docking and translocation of the nascent chain into the ER lumen commences (3, 4). After cleavage of the signal peptide in the lumen of the ER by signal peptidase, translocation of the polypeptide proceeds (reviewed in Ref. 5).

Most type II proteins ($\text{N}_{\text{cyt}}\text{C}_{\text{exo}}$) possess an uncleaved hydrophobic domain near the amino terminus that serves as both a signal sequence and an anchor domain (6). Following targeting of a type II nascent protein chain and ribosome to the ER membrane by SRP, the carboxyl terminus is translocated across the ER membrane while the amino terminus remains cytosolic. The charged amino acids that flank hydrophobic transmembrane (TM) domains are thought to play an important role in determining the ultimate orientation of the protein in the membrane. According to the “positive inside” rule of von Heijne, the flanking region with the greatest net positive charge is generally oriented facing the cytosol (7). A survey of a large number of integral membrane proteins confirmed this prediction, showing that the difference in charges of the 15 amino acids on either side of the first internal signal-anchor determines the topology of the mature protein (8). By mutating key charged residues that flank the TM domain of a type II viral protein, topology could be inverted to a type I orientation (9). The mechanism by which these charge differences are sensed at the ER membrane is not known (10).

Recently, a class of integral membrane proteins with carboxyl-terminal membrane anchors (CTMAs) was identified (11). This class includes a large number of proteins that are important for the targeting and fusion of intracellular vesicles (v- and t-SNARE (soluble NSF attachment protein receptor) proteins) (12), membrane-bound protein tyrosine phosphatases, cytchromes, and others (see Ref. 11). Proteins of this class are characterized by the presence of a carboxyl-terminal membrane spanning domain without an amino-terminal signal sequence and are oriented facing the cytosol. The mechanism by which CTMA proteins target to and insert in membranes has not been well studied.

In a recent study, we reported the cloning and initial characterization of a lymphoid-restricted protein called Jaw1 (13). Jaw1 has a coiled-coil domain in the middle-third of the protein and a carboxyl-terminal membrane anchor with a conserved 36-amino acid lumenal tail. Expression of Jaw1 mRNA and protein is developmentally regulated in both the B and T cell lineages with highest expression in pre-T, pre-B, and mature B cells. Several lines of evidence suggest that Jaw1 protein is expressed exclusively in the endoplasmic reticulum. The protein co-localized with the ER marker BiP in lymphocytes by indirect immunofluorescence and confocal microscopy. When transfected into Hela cells, which lack the protein, Jaw1 targeted to the ER as demonstrated by co-localization with the ER luminal protein, PDI. Furthermore, in an in vitro translation system the Jaw1 protein inserts into pancreatic microsomes and behaves as an integral membrane protein. Thus, these
data suggest that Jaw1 is a resident protein of the ER. Based on its expression pattern and localization to the ER, the protein may have a role in the developmentally regulated trafficking of the antigen receptors in B and T cells, antigen receptor assembly, or may otherwise influence lymphoid development.

In this report we provide evidence that the ER localization domain of Jaw1 resides within the carboxy-terminal 71 amino acids containing the membrane anchor. In addition, we describe a novel post-translational processing event at the carboxy-terminal of the Jaw1 protein upon insertion of the protein into microsome membranes and demonstrate that Jaw1 associates with the ER membrane post-translationally. These findings have implications for the targeting and membrane insertion of the class of proteins that contain carboxy-terminal membrane anchors.

**EXPERIMENTAL PROCEDURES**

**Constructs**—The full-length mouse Jaw1 cDNA was described previously (13). For the CMPK-Jaw1 fusion construct, PCR was used to amplify the region from His-468 to Val-539 of the mouse Jaw1 cDNA with BamHI 5’ and EcoRI 3’. This fragment was cloned into the BamHI and EcoRI sites of the CMPK expression plasmid pSPK (14) that contains amino acids 17–476 of CMPK.

To generate the full-length mouse Jaw1 (J1) and TM-deleted (J1 TM(--)) templates, PCR was used to engineer an SP6 RNA polymerase site and a strong β-globin ATG and leader sequence at the 5’ end of each template. The sequence of the forward primer for both constructs was CTATTTGGTGACACTTAAAACTGATCTGCGTCCGTGTTAAAAAGTTCCGG, with the last 18 bases corresponding to Jaw1 sequence beginning at amino acid 2. The reverse primer for the full-length template amplified the full-length coding region of the mouse Jaw1 cDNA including the termination codon, whereas the TM-deleted template ended at amino acid 479. Amplified products were gel-purified and used directly for transcription reactions. To construct the coiled-coil deleted FLAG-epitope Jaw1 construct (Jaw1-FLAG), the carboxy-terminal of Jaw1 from amino acids 334–539 was amplified by PCR with 5’ HindIII and NotI sites and a 3’ BamHI site. This fragment was cloned into the HindIII and BamHI site of pSP72 (Promega). The amino terminus from amino acids 2–193 was then amplified with 5’ HindIII and Nhel site and a reverse primer that added the FLAG epitope onto the extreme carboxyl terminus of Jaw1 and a new stop codon, and a BamHI cloning site. This product was gel-purified and cloned into the XhoI and BamHI sites of PCG (15). This PCG construct, used originally for other unpublished experiments, was amplified with a 5’ primer corresponding to amino acids 2–7 with a 5’ NotI site and a reverse primer that added the FLAG epitope onto the extreme carboxyl terminus of Jaw1 and a new stop codon, and a BamHI cloning site. This product was gel-purified and cloned into the XhoI and BamHI sites of the pEpi in vitro translation vector, which provides a strong translation initiation site and a TK leader sequence. All engineered constructs were sequenced to ensure fidelity of PCR.

**Transfections and Immunofluorescence**—Hela cells were grown on sterile glass coverslips in a 100-mm culture dish and were transfected with sterile glass coverslips in a 100-mm culture dish and were transfected with

**Subcellular Fractionation and Western Blotting**—One liter of the B cell line WEHI 231 was grown in RPMI, 10% fetal calf serum in log phase to a density of 9 × 10⁶ cells/ml. The cells were washed once in ice-cold PBS, and the cell pellet was suspended in 5 ml of hypotonic lysis buffer (buffer A, 10 mM HEPES-KOH, pH 7.5, 10 mM KAc, 7.5 mM MgOAc, 1.5 mM dithiothreitol, 10 μM aprotinin, 10 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride) and incubated on ice for 10 min. All subsequent steps were performed at 4°C. The suspension was homogenized with 15 strokes in a 7.5-ml Dounce vessel with an A pestle. Approximately 70% lysis was confirmed by light microscopy. Nuclei and nonlysed cells were removed with a 2,000 × g centrifugation for 3 min at 4°C. The salt buffer concentration of the postnuclear supernatant was then adjusted to 50 mM HEPES-KOH, pH 7.5, 150 mM KAc, 5 mM MgOAc, 1 mM dithiothreitol (buffer B), and 0.25 mM sucrose, and the volume was increased to 13.5 ml. A 2.25-ml cushion of 2.0 mM sucrose in buffer B was overlaid with 2.25 ml of 1.3 mM sucrose/buffer B cushion in Beckman 7/16 × 5 inch centrifuge tubes. Finally, this was overlaid with 6.75 ml of postnuclear supernatant in 0.25 mM sucrose/buffer B. Duplicate samples were collected as a 1 ml sample in a swinging bucket rotor at 4°C and 28,000 rpm (approximately 140,000 × g) for 3 h. There were two distinct bands at both the 0.25/1.3 (1a and 1b) and 1.3/2.0 (2a and 2b) interfaces. The upper band at the 1.3/2.0 mM sucrose interface (interface 2a), expected to be enriched for rough ER, was heavy and brown in color. The various gradients and interfaces were removed with a pipetman, and the fractions from duplicate samples were combined.

Equivalent aliquots (1/500) of the postnuclear supernatant, each sucrose gradient, and interface were analyzed by immunoblotting. A 10% SDS-PAGE gel was transferred to nitrocellulose by electroblotting. The blot was blocked in TBS (Tris-buffered saline, 50 mM Tris 7.5, 150 mM NaCl) with 1% fraction V bovine serum albumin, 4% Carnation’s dry, low fat milk, and 0.1% Triton for 2 h, and then washed in TBS/0.3% Triton. The blot was incubated with affinity purified rabbit-anti-Jaw1 antibodies (200 ng/ml) in blocking buffer for 1 h, and washed in TBS/0.3% Triton. For detection, the blot was incubated for 1 h with horse-radish peroxidase-conjugated goat anti-rabbit Abs (Bio-Rad) at a 1:1,000 dilution in blocking buffer. Following washing in TBS/0.3% and a rinse in TBS alone, the blot was developed using enhanced chemiluminescence (Amersham Corp.) according to the manufacturer’s suggested protocol. The blot was then stripped and incubated with a 1:2000 dilution of a MAb against ribophorin I (kindly provided by G. Kreibich, New York University) followed by 1:5000 dilution of horseradish peroxidase-conjugated goat anti-mouse Abs (Bio-Rad) and enhanced chemiluminescence. Densitometry of the blots was performed as described (17).

**In vitro Translation**—Both the mouse and human full-length cDNAs were linearized with XhoI, and in vitro RNA was transcribed using T3 polymerase (Stratagene). The Jaw1-FLAG construct was linearized with BamHI and RNA was transcribed using SP6 polymerase. SP6 was also used to transcribe RNA from the J1 and J1TM(--)-gel-purified templates. The c-erbA α1 cDNA (kindly provided by K. Strait and J. Oppenheimer, University of Minnesota) was linearized with BamHI and in vitro RNA transcribed using T7 polymerase. In vitro translations were performed typically in a volume of 15 μl, with 5 μl of rabbit reticulocyte lysate (Life Technologies, Inc.), 1 μl of RNA (1 μg/ml), 1 μl of Met and 1 μl of ATP Translation mixture (mMahirin, Life Technologies, Inc.), 25 μl of [35S]methionine (~800 Ci/mmol, 10–15 mCi/ml, Amersham Corp.), and DEPC-treated H2O, according to the manufacturer’s suggested protocol. Commercially prepared canine pancreatic microsomes (Promega) were added at the beginning of the translation or after the translation with or without 1 mM cold methionine. To pellet the microsome membranes, the translated lysates were diluted to 25 μl with IVT dilution buffer (140 mM KAc, 2.5 mM MgOAc, 1 mM dithiothreitol, 50 mM HEPES-KOH, pH 7.5) and carefully layered over a 100-μl cushion of 0.5 mM sucrose in IVT dilution buffer in airjuce tubes. Samples were spun for 3 min at 29 p.s.i. in a Beckman airjuce at room temperature, and supernatants were precipitated with 10% trichloroacetic acid. Membrane pellets and precipitated supernatant proteins were solubilized in 2 × SDS loading buffer with 5% 2-mercaptoethanol, and analyzed by SDS-PAGE. The gels were fixed in 40% methanol, 10% acetic acid for 20 min at room temperature and soaked in Amplify (Amersham Corp.) before fluorography.

**Immunoprecipitations**—For metabolic labeling experiments, the 38B9 mouse pre-B cell line was grown in RPMI, 10% fetal calf serum and harvested while growing in log phase. Cells were washed in me-
thionine-free RPMI, resuspended at 5 × 10⁶ cells/ml in methionine-free medium supplemented with 5% dialyzed fetal calf serum and 10 mM HEPES-NaOH, pH 7.5, and then incubated for 15 min at 37°C to deplete intracellular methionine pools. Cells were then pelleted and metabolically labeled in methionine-free complete media supplemented with 100 μCi/ml in vitro translation grade [³⁵S]methionine (Amersham Corp.) for 10 min at 5 × 10⁶ cells/ml. Cold methionine was then added to a final concentration of 0.5 mM, and the cells were chased for varying lengths of time. Cells were then pelleted, washed in ice-cold PBS × 2, and lysed by the addition of 500 μl of RIPA lysis buffer (1% Triton, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.5, 150 mM NaCl, and protease inhibitors) per 5 × 10⁶ cells and incubated on ice for 30 min. Nuclei and insoluble proteins were removed by centrifugation at 14,000 × g for 15 min at 4°C. Lysates were pre cleared with 25 μl of protein A-Sepharose beads preloaded with 7 μl of normal rabbit sera overnight at 4°C on a rotator. The next day beads were pelleted, and the lysates were diluted 1:1 with ice-cold NET-gel (0.1% Triton, 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.25% gelatin, 0.02% azide) and were incubated with beads preloaded with either affinity purified anti-Jaw 1 Abs or normal rabbit sera for 4 h at 4°C on a rotator. Beads were then washed sequentially with NET-gel with 500 mM NaCl, NET-gel with 0.1% SDS, and finally with 10 mM Tris, 0.1% Triton. After careful removal of the last wash, immunoprecipitated protein on beads was solubilized with 40 μl of 1× SDS loading buffer, boiled for 10 min, loaded on 10% SDS-PAGE gels, and analyzed by fluorography. For immunoprecipitation of in vitro translated proteins, an identical protocol was used except that 1% Triton was the only detergent used in the lysis buffer. The M2 monoclonal Ab recognizing the FLAG epitope (IBL, Kodak) was preloaded on protein A beads and was used as described above, except that the NET-gel/0.1% SDS wash was omitted.

RESULTS

Jaw1 Targets to the ER via a 71-Amino Acid Carboxyl-terminal Domain—We have previously shown that the Jaw1 protein co-localizes with the ER marker BiP in lymphocytes, targets to the ER when transiently transfected into HeLa cells, and is oriented on the cytosolic face of the ER (13). Based on the structure of the cDNA and its association with ER membranes in vitro (13), we hypothesized that the carboxyl terminus of Jaw1, which contains a TM domain, was the targeting signal that directed the protein to the ER (see Fig. 1A for a diagram of the full-length cDNA). To test this, a fragment of the mouse Jaw1 cDNA encoding the carboxyl-terminal 71 amino acids of mouse Jaw1 was cloned downstream of a cDNA encoding a cytoplasmic protein, chicken muscle pyruvate kinase (CMPK), in an expression vector (see Fig. 1, CMPK-J1–71), and this construct was transiently transfected into HeLa cells. The CMPK-Jaw1 fusion protein (Fig. 2A) co-localized with the ER protein PDI (Fig. 2B), whereas CMPK alone was localized diffusely throughout the cytosol (Fig. 2C). Co-localization of the CMPK-Jaw1 protein with PDI was confirmed by computer merging of the confocal images (not shown). A fusion protein of CMPK plus the carboxyl-terminal 150 amino acids of Jaw1 also targeted to the ER, whereas a fusion of CMPK with the 36 luminal amino acids of Jaw1 remained cytosolic (data not shown). In other experiments, overexpression of mutant Jaw1 proteins that remove the carboxyl-terminal 71 amino acids resulted in a cytosolic distribution of the protein as determined by both immunofluorescent staining and immunogold electron microscopy. Thus, the carboxyl-terminal 71 amino acids of Jaw1, which contain the TM domain, are both necessary and sufficient for targeting to the ER.

It was shown previously that Jaw1 protein translated in vitro in the presence of canine microsomes associated tightly with the microsomal membranes and could not be extracted with either high ionic strength or high pH, consistent with its being an integral membrane protein (13). We next investigated the behavior of a TM-deleted mutant of Jaw1 in an in vitro translation (IVT) system. Both full-length and TM(−) Jaw1 cDNAs (see Fig. 1A) were cloned into an IVT vector that contributed a strong translation initiation site resulting in a single translated protein species (in contrast with the multiple species observed when full-length Jaw1 cDNAs containing 5′- and 3′-untranslated regions and the native initiator methionine were translated, see below). The mutant IVT Jaw1 protein (J1TM(−)) did not pellet with the microsomal membranes but instead partitioned exclusively in the supernatant fraction.
This mutant behaved identically to c-erbA, a protein that does not associate with membranes (Fig. 3, lanes 5 and 6), but in contrast to wild-type Jaw1 (Fig. 3, lanes 1 and 2). Taken together, these data suggest that the carboxyl-terminal hydrophobic region of Jaw1 functions as a TM domain that anchors Jaw1 in the ER membrane.

**Jaw1 Protein Isoforms**—It was of interest that the full-length Jaw1-translated protein (Fig. 3, lanes 1 and 2) partitioned into both the pellet and supernatant, with a slowly migrating full-length form of the protein predominating in the soluble fraction (lane 2), and a faster migrating form of the protein found only in the pellet (lane 1). One potential explanation for this finding was that a processing event occurs upon stable insertion of the Jaw1 protein into microsome membranes. To investigate this we took a biochemical approach.

In an initial series of experiments, the cellular organelles of the WEHI 231 mouse B lymphocyte cell line were fractionated over discontinuous sucrose gradients. Following lysis and homogenization of the cells, a postnuclear supernatant was centrifuged over a 0.25/1.3/2.0 M sucrose step gradient, and the gradients and interfaces were analyzed by immunoblotting. As shown in Fig. 4A, lane 1, the postnuclear supernatant contained four major isoforms of Jaw1, designated A, B, C, and D ranging in size from 57 to 69 kDa as noted previously (13). In addition, other immunoreactive bands of approximately 42 and 46 kDa were seen. The 0.25 M sucrose gradient following centrifugation should contain soluble protein and was enriched for the A isoform of Jaw1 (lane 2). Isoforms B and D, and to a lesser extent the C isoform of Jaw1, were found at interface 2a (lane 7), in the expected migration position of the rough ER (RER). Lane 8 contains 1/10 of the material in lane 7, suggesting that this interface was approximately 10-fold enriched for Jaw1 protein compared with the other gradients and interfaces. Inverse 2a was similarly enriched for the RER type I membrane protein ribophorin I (lower panel, Fig. 4A), and this was confirmed by densitometry of both the Jaw1 and ribophorin I blots (Fig. 4B). These data suggest that Jaw1 is expressed as four major protein isoforms (A–D), with the A and, to a lesser extent, the C isoforms preferentially soluble (Fig. 4A, lane 2), whereas the B and D isoforms were enriched in the membrane-bound microsome fraction (lane 7).

**Jaw1 Processing**—To investigate the inter-relationship of the various Jaw1 protein isoforms, a pulse-chase immunoprecipitation was performed. Following a 10-min labeling period with [35S]methionine, 38B9 B lymphoid cells were chased with cold methionine, lysed at various time points, and immunoprecipitated with affinity purified rabbit polyclonal antibodies to Jaw1 (19). As shown in Fig. 5, the processing of isoforms A and

**Fig. 3.** The carboxyl-terminal region of Jaw1 is required for membrane association in vitro. Full-length Jaw1 (J1), TM-deleted Jaw1 (J1 TM(−)), or c-erbA a1 (c-erbA) were IVT in the presence of microsomes, and the intact microsomes were pelleted (P). Supernatants (S) were precipitated with trichloroacetic acid, and proteins in both pellets and supernatants were analyzed by SDS-PAGE.

**Fig. 4.** Jaw1 is enriched in microsomes by subcellular fractionation. A, the B lymphocyte cell line WEHI 231 was homogenized, and subcellular organelles were fractionated on a 0.25/1.3/2.0 M sucrose step gradient. 1/500 of each of the following preparatory fractions, gradients, and interfaces were electrophoresed through an SDS-PAGE gel, electroblotted, probed with affinity purified rabbit anti-Jaw1 antibodies (top panel), and developed using enhanced chemiluminescence. Lane 1, postnuclear supernatant; lane 2, 0.25 M sucrose load gradient; lane 3, 1.3 M sucrose gradient; lane 4, 2.0 M sucrose gradient; lane 5, interface 1a at 0.25/1.3 M sucrose interface; lane 6, layer 1b at 0.25/1.3 M sucrose interface; lane 7, layer 2a at 1.3/2.0 M sucrose interface; lane 8, 1/10th of the protein loaded in lane 7; lane 9, layer 2b at 1.3/2.0 M sucrose interface; and lane 10, control cytoplasmic lysate of WEHI 231. In the lower panel, the same blot was reprobed with a mAb to the luminal domain of the RER protein, ribophorin I. B, densitometry of the blots shown in A was performed as described (17).
C into isoforms B and D, respectively, is clearly demonstrated. This processing event proceeds to completion over a period of 3–4 h (compare with the pattern on Western blot, Fig. 4A)

FIG. 5. Pulse-chase immunoprecipitation of Jaw1 from a lymphoid cell line. 38B9 cells (5 × 10^6 cells/lane) were pulsed for 10 min with [35S]methionine and then chased for the indicated times in complete medium before lysis in RIPA buffer and immunoprecipitation with the Jaw1 antibodies. Isoforms A and C, 69 and 61 kDa, respectively, chase over time to the B and D isoforms, 65 and 57 kDa.

FIG. 6. In vitro translated Jaw1 undergoes processing in the presence of canine ER microsomes. A, full-length human (lane 1) and mouse (lane 2) Jaw1 cDNAs were IVT using rabbit reticulocyte lysates resulting in six major translation products, α–f, an antisense mouse Jaw1 control translation is shown in lane 3. B, addition of canine ER microsomes co-translationally to the mouse Jaw1 IVT results in mobility shifts of each of the multiple internal translation initiation isoforms of Jaw1 (α′–f′). The conserved methionines (number refers to amino acid) in the mouse Jaw1 cDNA and their predicted molecular masses are as follows: Met-1, 59.6 kDa; Met-44, 54.9 kDa; Met-97, 49.2 kDa; Met-281, 29.2 kDa; Met-288, 27.3 kDa; and Met-308, 26.0 kDa. We observed a slower migration of the Jaw1 protein isoforms, both from cells and IVTs, than would be expected from predicted molecular masses.

A proteolytic event removing the short luminal domain following membrane insertion of the Jaw1 protein.

For subsequent experiments, a Jaw1 IVT template was constructed that deleted the coiled coil region (140 amino acids), provided an optimized translation initiation codon at the 5′ end, and added the FLAG epitope (recognized by the M2 MAb-IBI/Kodak) onto the extreme carboxyl terminus of the protein (Jaw1-FLAG, see Fig. 1B). The internal deletion in this J1-FLAG construct was required in order to unequivocally follow the fate of the processed Jaw1 protein. Identical results were obtained with the full-length protein; however, the mobility shifts were more difficult to resolve on SDS-PAGE (Fig. 3 and data not shown). To directly test the hypothesis that the processing event removed the luminal domain of Jaw1, the Jaw1-FLAG construct was translated either without (Fig. 7; lanes 1, 3, and 5) or with canine microsomes (lanes 2, 4, and 6). Each lysate was then split into three aliquots and immunoprecipitated with either normal rabbit sera (lanes 1 and 2), the Jaw1 antibodies that recognize the amino terminus of the protein (lanes 3 and 4), or the M2 mAb that recognizes the luminal FLAG epitope (lanes 5 and 6). While the Jaw1 Abs immunoprecipitated both the full-length and processed versions of the protein (lane 4), the anti-FLAG Ab only immunoprecipitated the full-length protein (lane 6). This demonstrated that the carboxyl terminus of Jaw1 downstream of the TM domain was no longer recognized in the mobility shifted version of the protein, compatible with a proteolytic event. The decreased intensity of the bands in the FLAG immunoprecipitations (lanes 5 and 6) reflects an increased detergent sensitivity of the anti-FLAG antibody compared with the Jaw1 antibodies (lanes 3 and 4). Efforts to identify a proteolyzed fragment using either Western blotting with the anti-FLAG Ab or identification of the radiolabeled peptide on high percentage SDS-PAGE have not been successful, possibly due to extensive proteolysis of the fragment.

Processing of Jaw1 Occurs Post-translationally—Finally, based on the primary structure of Jaw1, with a carboxyl-terminal TM domain that should not emerge from the ribosome
until translation is complete (18), we tested whether Jaw1 associated with the microsome membranes post-translationally. The addition of cold methionine (final concentration 1 mM) at the initiation of the translation of Jaw1-FLAG resulted in the absence of labeled protein (Fig. 8, lane 1). The post-translational addition of microsomes either in the absence (lane 3) or presence (lane 5) of cold methionine resulted in the mobility shift, suggesting that Jaw1 is able to associate with membranes post-translationally. Of interest, if ribosomes were removed from the rabbit reticulocyte lysate translations by ultracentrifugation prior to addition of microsomes, neither the mobility shift nor membrane insertion was observed (not shown). This suggests that ribosomes or ribosome-associated proteins may be required for the post-translational membrane targeting. In other data not shown, neither the mobility shift nor membrane insertion could be demonstrated in standard wheat germ lysates (3), suggesting the possibility that the protein or proteins responsible for post-translational targeting of Jaw1 protein to the ER in rabbit lysates are missing or deficient in the wheat germ system.

**Discussion**

In this report we describe the targeting of the lymphoid-restricted protein Jaw1 to the ER via a short carboxyl-terminal region that contains a TM domain. The carboxyl-terminal 71 amino acids of Jaw1 were able to confer ER localization to the normally cytosolic protein CMKP (Fig. 2), suggesting that all of the targeting information required was localized to this short domain. In complementary *in vitro* translation experiments, removal of the carboxyl-terminal hydrophobic domain of Jaw1 prevented its association with microsomal membranes. These results suggest that the carboxyl terminus of Jaw1 is both necessary and sufficient for targeting the protein to the ER.

Relatively few studies have examined the problem of ER targeting and retention of type II proteins or CTMA proteins. Two yeast type II membrane proteins contain the sequence HDEL at their lumenal carboxyl termini and are retrieved by the HDEL receptor system similar to many soluble ER proteins (19). Protein tyrosine phosphatase 1B targets to the ER via a hydrophobic 35-amino acid domain, and this region was able to confer ER localization on the normally cytosolic heterologous protein CMKP (20). However, the region responsible for ER targeting of protein tyrosine phosphatase 1B does not contain a typical membrane spanning domain, and the protein is partially extracted from membranes at high pH suggesting that protein tyrosine phosphatase 1B may not be an integral membrane protein (20). The ER targeting of the CTMA protein cytochrome b5 was recently reported, and interestingly only a 10-amino acid hydrophilic luminal domain was necessary for conferring ER localization to a heterologous protein (21).

A di-basic motif at positions −3, and −4/−5 from the carboxyl terminus of some type I ER TM proteins serves as a retention/retrieval signal for the ER (22–24). Initially observed in the viral protein E3/19K and the resident ER protein UDP-glucuronosyltransferase, this motif confers ER localization when transferred to chimeric CD8 or CD4 proteins. A similar motif was found in a CD4 truncation mutant (25), transfected T cell receptor γ and δ chains retained in the ER (26), a membrane protein of the yeast ER and nuclear envelope (27), and in a protein involved in polypeptide ER translocation (28).

The region of Jaw1 shown to confer ER localization consists of 11 amino acids amino-terminal to the TM domain (the TM flanking region), the 24 amino acids of the TM domain itself, and the 36-amino acid luminal tail. The luminal tail alone was not sufficient to target CMKP to the ER (data not shown). This, together with the finding that Jaw1 undergoes a processing event shortly after insertion into the ER membrane that likely removes the luminal tail, suggests that the novel ER retention/retrieval motif of Jaw1 resides either in the TM domain or in the 11 amino acids of the TM flanking region. There is a consensus motif, BBXXB (where B is a basic residue), present in the TM domain flanking region of both human and mouse Jaw1 proteins, as well as two unrelated ER proteins with CTMAs, PIG-A, a protein required for an early step in the biosynthesis of glycosylphosphatidylinositol membrane anchors and shown to be the gene defective in the human disease paroxysmal nocturnal hemoglobinuria (29), and UBC6, a component of the ubiquitin degradation system in yeast (30). The same motif, with an additional lysine, is also present in the BOS1 gene of yeast (31). The presence of a BBXXB motif in the TM domain flanking region of these unrelated resident ER CTMA proteins is provocative and is reminiscent of the di-basic motif found near the cytosolic tail of certain type I resident ER proteins shown to be important for retention/retrieval to the ER (22).

We extend our previous results on the ER localization of Jaw1 by demonstrating that Jaw1 co-sediments with the RER marker Ribophorin I on discontinuous sucrose gradients. These experiments also demonstrated differential sedimentation of the various Jaw1 protein isoforms with the A isoform of Jaw1 predominantly soluble, whereas the B and D isoforms of Jaw1 were microsome-bound (Fig. 4A). Immunoprecipitations clearly demonstrated the post-translational processing of the A and C isoforms of Jaw1 to the faster migrating isoforms B and D over approximately 3 to 4 h (Fig. 5B). A similar processing event was observed with *in vitro* translated Jaw1 in the presence of canine microsomes (Fig. 6). By immunoprecipitating the IVT protein with antibodies raised against the amino terminus of the protein or antibodies recognizing an engineered epitope tag in the luminal domain, it was shown that the processing event removes the luminal domain. Previously well characterized...
proteolytic events in the ER lumen are limited to the cleavage of signal peptides by signal peptidase and the degradation of proteins or unassembled oligomeric proteins. Yuk and Lodish (32) reported an ER luminal cleavage event in the type II protein human asialoglycoprotein receptor at a site close to the transmembrane domain prior to protein degradation in the ER. The specificity of the protease responsible for cleavage of the asialoglycoprotein receptor was shown by mutagenesis to be similar to that of signal peptidase. The protease responsible for Jaw1 cleavage remains to be determined. Of interest, we also note a conserved potential signal peptidase cleavage site in the luminal domain of Jaw1 six residues downstream of the hydrophobic domain (QKSVD/A/A)PTQQEDSW . . . (human) and QTA VE/A/A)PTQEGDSW . . . (mouse) with the / denoting the potential cleavage site, small uncharged amino acids at −1 and −3 from the putative cleavage site (A and V, respectively, underlined), and a bulky charged group at −2 (D or E) (33).

This study also demonstrates that the targeting of Jaw1 to the ER occurs post-translationally. It is likely that all CTMA proteins target to their respective compartments in a similar post-translational fashion. With the first residue of the membrane anchor of Jaw1 located only 60 amino acids from the penultimate carboxyl-terminal residue, translation of the Jaw1 polypeptide should terminate before the anchor region emerges from the ribosome (18). It follows, therefore, that SRP, which binds to the nascent chain while still ribosome bound, is unlikely to participate in the targeting of Jaw1 to the ER membrane (34). In support of this, the post-translational targeting of one member of the CTMA class of proteins, cytochrome b5, has previously been demonstrated to be independent of SRP (35), and the yeast syntaxin homologue Sso2p associates with dog pancreatic microsomes post-translationally (36). The hydrophobic domain at the carboxyl terminus of cytochrome b5 has been referred to as an insertion sequence to distinguish it from an SRP-dependent signal sequence (35).

We propose the following model for ER membrane association and processing of Jaw1. Shortly after translation of Jaw1, the protein targets to the ER via targeting determinants present either within the TM domain or the TM domain flanking region. This targeting requires either ribosomes or ribosome-associated proteins and is unlikely to involve SRP. The carboxyl-terminal 36 amino acids 3′ of the TM domain of Jaw1 are then translocated into the lumen of the ER where proteolytic removal of this short domain occurs. Although the data shown do not directly address the issue of which side of the membrane the cleavage event occurs, fusion of an immunogenic flu peptide to the luminal domain of Jaw1 results in antigen presentation of the peptide when the cDNA is overexpressed in a TAP1-deficient cell line.3 The functional significance of this cleavage event is not yet understood. We speculate that the luminal domain of Jaw1 may be transiently required for the protein to associate with either itself or other proteins, and/or this domain may serve a role in redistributing the protein to a subcompartment of the ER (37). A better understanding of the function of this protein may allow us to explore these possibilities.

This study raises a number of interesting questions regarding the targeting of CTMA proteins to intracellular compartments. Since these proteins are all likely to target to their respective compartments post-translationally, the nature of the molecular recognition system that directs these proteins to their appropriate intracellular membranes remains to be determined. It is possible that it yet to be identified cytosolic chaperones might play a role both in the folding of the protein in the cytosol as well as recognizing the targeting signals and directing the proteins to membranes. A subclass of the CTMA proteins, the SNAREs, participate in vesicle fusion events throughout the cell, and the targeting of these proteins to their appropriate intracellular location must be very precise to avoid untoward vesicle fusion events within the cell. Based on our observation that the targeting and membrane insertion of Jaw1 is deficient in wheat germ lysates, it may be possible to identify biochemically the components of rabbit reticulocyte lysates that complement the wheat germ system in Jaw1 targeting.

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