Two Upstream Cysteines and the CAAX Motif but Not the Polybasic Domain Are Required for Membrane Association of Xlcaax in *Xenopus* Oocytes†

Malgorzata Kloc, Xiao Xia Li, and Laurence D. Etkin*

Department of Molecular Genetics, University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, Texas 77030

Received January 29, 1993; Revised Manuscript Received April 14, 1993

ABSTRACT: We have analyzed the role of several protein motifs in controlling the membrane association of the xlcaax-1 protein in *Xenopus* oocytes. Xlcaax-1 is a maternally expressed protein that during development is associated with the basal lateral membrane of polarized epithelial cells. It is enriched in the tubule cells of the adult kidney and several other organs that are involved in osmoregulation. Xlcaax-1 has a C-terminal CAAX sequence (CVVM) identical to that of N-ras, followed by two cysteines that are potential palmitoylation sites and a polybasic domain. Mutants were constructed that either deleted specific domains or changed specific amino acids of the consensus sequences in or near the CAAX motif. Synthetic mRNAs were injected into *Xenopus* oocytes and their protein products analyzed for their ability to associate with the oocyte plasma membrane. A mutation changing cysteine-588 of the CAAX box to serine or the inhibition of prenylation by lovastatin eliminated the membrane association of the protein. Mutation of either of the upstream cysteines (either 585 or 587) also inhibited the association of xlcaax-1 with the membrane. Unlike Ras, however, deletion of the polybasic domain had no effect on membrane binding. In addition, we show that xlcaax-1 binds ATP but not GTP.

We have recently described a unique maternally expressed 110-kDa protein, xlcaax-1, that is localized to the plasma membranes of *Xenopus* oocytes and XTC cells (Kloc et al., 1991). During development, xlcaax-1 protein is found in the basolateral membranes of kidney tubules and other polarized epithelia and is associated with the ion-transporting membranes in several adult organs of *Xenopus* (Cornish et al., 1992). The localization pattern of xlcaax-1 suggests that it may operate in conjunction with an ion transport channel or pump and that its attachment to the plasma membrane may be required for its proper function.

The xlcaax-1 protein belongs to the functionally diversified group of membrane-associated proteins such as ras, the ras-related family, nuclear lamins, G25K protein, and the γ subunit of transducin [Hancock et al., 1989; Vorburger et al., 1989; Kitten & Nigg, 1991; Maltese & Sheridan, 1990; Hurley et al., 1984; for reviews, see Khosravi-Far et al. (1992) and Nigg et al. (1992)]. All of these proteins contain a C-terminal motif commonly referred to as the CAAX box (C = cysteine, A = aliphatic, X = any amino acid). As shown conclusively for several of these proteins, the CAAX tetrapeptide specifies a series of posttranslational modifications (addition of an isoprenyl group, proteolytic cleavage of the last three amino acids, and carboxymethylation of the C-terminal cysteine) that are responsible for the association of the proteins with cellular membranes (Hancock et al., 1989, 1991; Kitten & Nigg, 1991; Vorburger et al., 1989; Gutierrez et al., 1989; Fujiyama et al., 1990; Jackson et al., 1990; Kato et al., 1992). In addition, some of the CAAX proteins such as H, N, and Kras4A contain a second membrane binding signal consisting of one or two cysteine palmitoylation sites. Also, some CAAX box proteins, such as Kras4B, possess a polybasic domain upstream of the CAAX motif [see Kloc et al. (1991)]. Mutational analysis showed that the combination of the CAAX motif and a second signal of either palmitoylation or a polybasic domain is required for the localization of different forms of p21 ras to the plasma membrane (Hancock et al., 1990, 1991).

The xlcaax-1 protein possesses a C-terminal CVVM sequence, which is identical to that of the N-ras protein, and two cysteine residues immediately upstream of the CAAX box (CQCCVVM). In addition to these two signals, xlcaax-1 has a third putative signal—a stretch of five positively charged basic residues (RRKKK) that is very similar to the polybasic domain in several nonpalmitoylated forms of the ras, rho, and rap families (Hancock et al., 1990; Kloc et al., 1991).

We have shown previously that xlcaax-1 undergoes isoprenylation and palmitoylation when expressed in the baculovirus system and is isoprenylated and palmitoylated in XTC cells (Kloc et al., 1991). We have also demonstrated that the protein is associated with the membrane fraction in an isoprenylation-dependent manner in XTC cells. In this study, we used mutational analysis involving specific deletions and amino acid substitutions to examine the role of the CAAX motif, two upstream cysteine palmitoylation sites, and the polybasic domain in the membrane association of xlcaax-1 in *Xenopus* oocytes. In addition, since xlcaax-1 has a potential nucleotide binding site, we tested the ability of the protein to bind ATP and GTP which will help establish its function in the cell.

MATERIALS AND METHODS

DNA Constructs and Mutagenesis. RNA transcripts were made from a construct containing the bluescript vector, the T3 promoter, the 5′ untranslated region of *Xenopus* β-globin gene, and a 12 amino acid long T7 viral coat protein tag. The xlcaax-1 cDNA was fused in-frame with the upstream T7 tag sequence (Li, Shou, Kloc, Reddy, and Etkin, submitted for publication). T7 coat protein antibody was subsequently used for the immunoprecipitation of the exogenous xlcaax-1 wild-type and mutant proteins. As another means to distinguish the exogenous xlcaax-1 protein from the endogenous protein,

† This work was supported by NSF Grant IBN-9007410 and NIH Grant RR-5511.

* Address correspondence to this author.

0006-2960/93/0432-8207$04.00/0

we made a truncated version of xlcaax-1 by deleting the sequences coding for the last 143 amino acids of the xlcaax-1 protein from the full-length cDNA. This deletion mutant was called xlcaaxΔ448–591. Subsequently, the wild-type or mutant oligonucleotides coding for amino acids 577–591 were added to the 3′ end of the xlcaaxΔ448–591 mutant. This construct possessing the wild-type oligonucleotide is referred to as xlcaaxΔ448–576 and lacks 128 amino acids in the middle of the cDNA. The sequences of all constructs were confirmed by DNA sequencing using Sequenase (USB).

In Vitro Transcription of RNA and Injection into Oocytes. Capped RNA was synthesized *in vitro* from the linearized wild-type and mutant DNA templates using the T3 MEGAscript *in vitro* transcription kit (Ambion) according to the manufacturer's specifications. *Xenopus* stage VI oocytes obtained from anesthetized frogs were defolliculated manually and injected with 20 ng (1 ng/nL) of RNA (Etkin & Maxson, 1980).

Oocyte Labeling. Injected oocytes were incubated in modified Barth's saline overnight at 18 °C in the presence of Trans ^{35}S label (labeled methionine and cysteine, specific activity 1197 Ci/mmol; ICN Biochemicals) at a final concentration of 1 mCi/mL. In some experiments, oocytes were incubated with [^{14}C]mevalonolactone (specific activity 55 Ci/mol; American Radiolabeled Chemicals Inc.) at a final concentration of 50 μCi/mL in the presence of lovastatin (gift from Dr. A. Alberts, Merck Sharp & Dohme). In pulse–chase experiments, oocyte incubation medium was supplemented with 2 mM unlabeled mevalonolactone (Sigma).

Membrane and Cytoplasm Isolation and Immunoprecipitation. Labeled oocytes were manually dissected into plasma membranes and cytoplasms. All manipulations were done on ice in the presence of AEBSF protease inhibitor cocktail (Calbiochem). In brief, batches of 10 injected oocytes were placed in small Petri dishes in 500 μL of membrane isolation buffer (Sadler & Maller, 1981). Membranes were peeled off the cytoplasms manually and collected in 1.5-mL tubes containing 500 μL of membrane isolation buffer. After being pelleted by centrifugation, membranes were washed twice in 1 mL of buffer. Final membrane pellets were homogenized in 1× RIPA buffer (150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, and 10 mM Tris-HCl, pH 7.2). Cytoplasms remaining after the membrane isolation were transferred to 1.5-mL tubes and centrifuged to remove yolk and contaminating membranes. The supernatant was adjusted to final concentration of 1× RIPA.

Membrane and cytosolic fractions were immunoprecipitated with a polyclonal antibody against the T7 coat protein (Novagen) at 1:500 final dilution. After 1-h incubation on ice, immunocomplexes were precipitated using protein A positive *Staphylococcus aureus* cells (BMB) and washed according to Kloc et al. (1991). Pellets were solubilized by boiling in loading buffer and separated by SDS–7.5% PAGE. The gels were fixed, fluorographed in AMPLIFY (Amersham Corp.), and exposed to X-ray AR5 film at –80 °C. To assess the amount of radioactivity in the immunoprecipitated protein bands, densitometric measurements of autoradiographs were done using a Ephortec Joyce Loebel densitometer.

ATP and GTP Binding Assays. Stage VI oocytes were injected with xlcaax or coinjected with xlcaax and xnf7 RNA and were incubated 16 h at 18 °C in 1× Barth's. Xnf7 was used as a negative control since it is a transcription factor that does not have any known nucleotide binding sites (Reddy et al., 1991). After homogenization and immunoprecipitation with T7tag antibody (see above), immunocomplexes were separated on 7.5% SDS–PAGE and transferred electrophoretically to nitrocellulose paper in 25 mM Tris/192 mM glycine/30% methanol.

Table I: Oocytes Injected with Different RNA Templates Were Labeled for 18 h with ^{35}S-Trans Label and Manually Dissected into Membranes and Cytoplasms[a]

construct	% of protein in cytoplasm	% of protein in membrane
full-length wild type	40	60
wild-type Δ448–576	40	60
deletion mutant Δ448–591	97	3
mutant Cys-588	97	3
mutant Cys-585	81	19
mutant Cys-587	89	11
mutant Cys-585 and -587	84	16
mutant, basic region deleted	40	60

[a] Labeled proteins from each fraction were immunoprecipitated using T7 antibody and resolved by SDS–PAGE and fluorography. The autoradiographs were quantitated by densitometric scanning. The proportion of membrane- or cytoplasm-associated xlcaax-1 is represented as a percentage of the total (membrane + cytoplasm) immunoprecipitated radioactivity. The numbers were derived from three to five experiments for each mutant.

The nucleotide binding assays were performed according to Lapetina and Reep (1987). The transfer blots were rinsed briefly with binding buffer (50 mM Tris-HCl, 0.3% Tween 20, 5 mM $MgCl_2$, and 1 mM EGTA, pH 7.5), preincubated 10 min in binding buffer at 25 °C, and incubated 90 min at 25 °C in binding buffer containing either [^{32}P]ATP (1 μCi/mL, specific activity 3000 Ci/mmol) or [^{32}P]GTP (1 μCi/mL, specific activity 3000 Ci/mmol). For competition experiments, blots were preincubated in the presence of 1 μM unlabeled ATP or GTP. After incubation, blots were washed in four changes of binding buffer (30 min each wash) at 25 °C, air-dried, and exposed to X-ray film at –80 °C.

RESULTS

Expression and Localization of Wild-Type Xlcaax-1. Our earlier study showed that in *Xenopus* oocytes endogenous xlcaax-1 protein is associated with the plasma membrane (Kloc et al., 1991). To assess the role of the CAAX motif and the secondary signals (upstream cysteines and polybasic domain) in the association of xlcaax-1 with the membrane, we injected *in vitro* synthesized RNA from the wild-type and mutated DNA templates into oocytes. RNA from full-length xlcaax-1 coding for the 591 amino acid xlcaax-1 protein and RNA from xlcaaxΔ448–576, which contains a 128 aa deletion in the middle of the cDNA, were injected into the cytoplasm of *Xenopus* oocytes. Both constructs possess the intact wild-type polybasic domain, two upstream cysteines, and the CAAX motif at their carboxy-terminal ends. After overnight incubation in the presence of [^{35}S]methionine, the membranes and cytoplasms were isolated from the injected oocytes, and exogenous xlcaax protein was immunoprecipitated using the T7 tag antibody (see Materials and Methods).

For both of these proteins, the pattern of association with the oocyte membrane was identical. The densitometry measurements of immunoprecipitated radioactive protein bands showed that between 55 and 74% of the xlcaax protein from both constructs colocalized with the membrane and between 26 and 45% with the oocyte cytosolic fraction (Table I and Figures 1 and 2). This pattern of localization was similar to that of endogenous xlcaax-1 protein [see Kloc et al. (1991)]. Deletion of the entire C-terminal region (xlcaaxΔ448–591) including the polybasic domain, the two upstream cysteines,

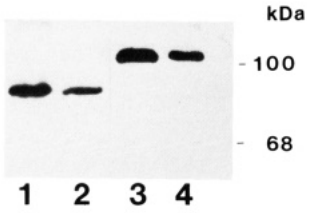

FIGURE 1: Localization of full-length and shortened wild-type constructs. Localization of shortened wild-type (lanes 1 and 2) and full-length wild-type (lanes 3 and 4) xlcaax protein in *Xenopus* oocytes. Both constructs were translated *in vivo* in stage VI oocytes from injected *in vitro* synthesized RNA templates. ^{35}S-Trans-labeled proteins from manually isolated oocyte membranes (M) and cytoplasms (C) were immunoprecipitated using antibody against T7 coat protein, analyzed by SDS–7.5% PAGE, and fluorographed. For both constructs, over 60% of labeled protein was present in oocyte membrane fractions (see also Table I).

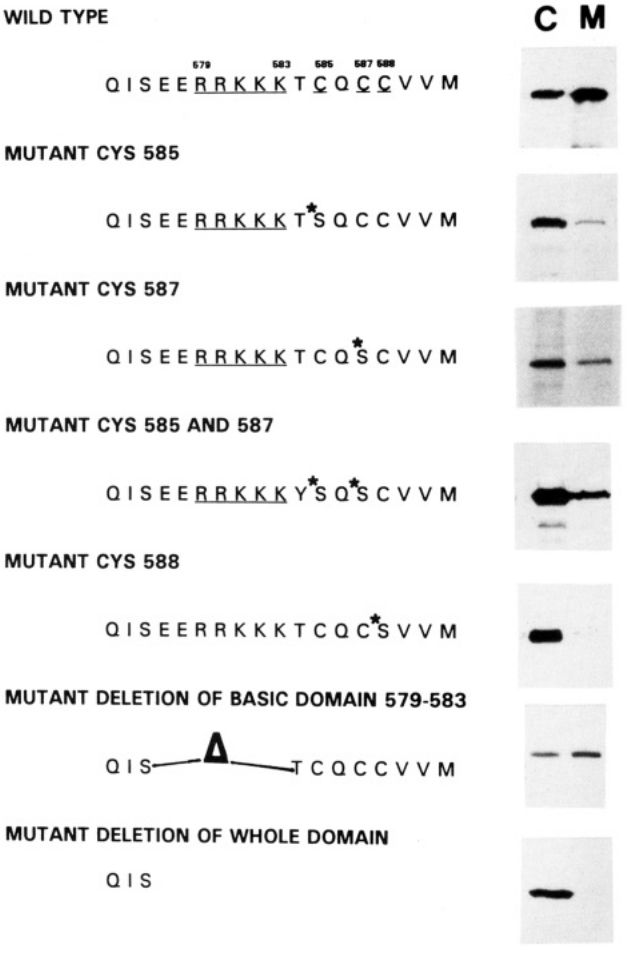

FIGURE 2: Effect of carboxy-terminal mutations on the localization of xlcaax-1 protein in *Xenopus* oocytes. The amino acid sequences of wild-type and mutant forms are given in the left panel. The numbers above the amino acid depict its position in the full-length xlcaax protein. Substitution of serine for cysteine is marked by the amino acid symbol and star. The wild-type protein is xlcaaxΔ448–576, which has an intact, wild-type carboxy terminus from aa 576 to 591 reinserted back into the molecule. Wild-type and mutant forms of xlcaax-1 protein were translated *in vivo* in *Xenopus* oocytes from the injected synthesized *in vitro* RNA templates in the presence of ^{35}S-Trans label. Proteins were immunoprecipitated using polyclonal antibody against T7 coat protein and analyzed by SDS–7.5% PAGE and fluorography. The right panel shows the bands of labeled proteins immunoprecipitated from cytoplasms (C) or manually isolated membranes (M) of *Xenopus* oocytes.

and the CAAX box completely eliminated membrane association (Table I). In the next experiments, we used the xlcaaxΔ448–576 construct and hereafter refer to it as the

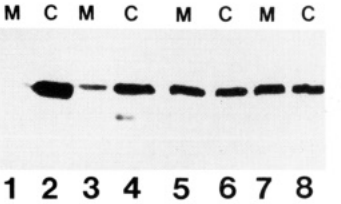

FIGURE 3: Membrane association of xlcaax-1 in oocytes is prenylation dependent. *Xenopus* oocytes injected with RNA synthesized *in vitro* from wild-type template were incubated in the presence of ^{35}S-Trans label and 50 μM lovastatin. After 18 h, some of the oocytes were dissected into membranes and cytoplasms, and proteins were immunoprecipitated with T7 antibody (lanes 1 and 2). The rest of the oocytes was postincubated in the fresh medium without lovastatin and label but supplemented with 2 mM unlabeled mevalonolactone. Membranes and cytoplasms were isolated and immunoprecipitated after 12 h (lanes 3 and 4), 24 h (lanes 5 and 6), and 48 h (lanes 7 and 8) of incubation. All immunoprecipitates were analyzed by SDS–7.5% PAGE and fluorography. This was a 16-h exposure.

wild type since it behaves like the endogenous protein with respect to membrane association.

Association of Xlcaax-1 with the Oocyte Plasma Membrane Is Isoprenylation-Dependent. The CAAX motif directs isoprenylation of ras, ras-related proteins, yeast a-mating factor, and nuclear lamins (Hancock et al., 1989; Kitten & Nigg, 1991; Vorburger et al., 1989; Marcus et al., 1991). Previously we showed that xlcaax-1 (which contains the CVVM motif) is also isoprenylated when expressed in the baculovirus system and its membrane association in *Xenopus* XTC cells is prenylation-dependent (Kloc et al., 1991). Using pulse–chase experiments, we investigated whether the wild-type xlcaax undergoes isoprenylation in *Xenopus* oocytes and whether its association with the oocyte plasma membrane is isoprenylation-dependent.

Oocytes injected with *in vitro* synthesized wild-type RNA were incubated with [^{35}S]methionine in the presence of lovastatin, which inhibits the synthesis of endogenous mevalonic acid and depletes the cell of endogenous isoprenoids. After overnight incubation, some of the oocytes were dissected into membranes and cytoplasms and the fractions processed for immunoprecipitation. The remaining oocytes were postincubated for 12, 24, and 48 h in fresh medium lacking lovastatin or [^{35}S]methionine but supplemented with 2 mM unlabeled mevalonolactone. At 0, 12, 24, and 48 h, membranes and cytoplasms were isolated, and ^{35}S-labeled proteins were immunoprecipitated using the T7 epitope tag antibody and analyzed by SDS–PAGE and fluorography.

Figure 3 shows that after overnight incubation in the presence of lovastatin all labeled xlcaax-1 protein was localized in the oocyte cytoplasm (lanes 1 and 2). The removal of the lovastatin from the incubation medium allowed the protein to associate slowly, over the period of 24 h, with the oocyte plasma membrane (lanes 3–6). These results suggest that, as in the case of other isoprenylated proteins (Kitten & Nigg, 1991; Hancock et al., 1991; Kato et al., 1992) as well as xlcaax in *Xenopus* XTC cells (Kloc et al., 1991), the association of xlcaax-1 with the oocyte plasma membrane is isoprenylation-dependent.

We were also able to demonstrate directly that xlcaax is isoprenylated in oocytes using a biochemical approach. Oocytes were injected with *in vitro* synthesized wild-type RNA and incubated with [^{14}C]mevalonate, which is the precursor of isoprenoids. To increase the incorporation of labeled precursor into the protein, oocytes were depleted of endogenous isoprenoids by incubation with lovastatin. After overnight incubation, oocytes were transferred into the fresh medium

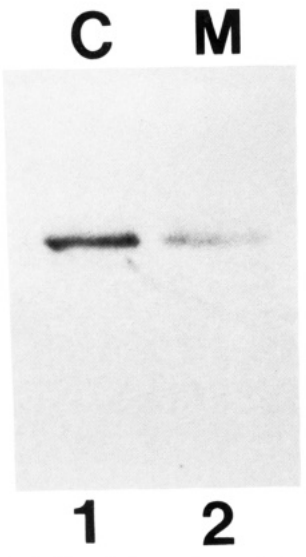

FIGURE 4: Isoprenylation of xlcaax-1 protein in *Xenopus* oocytes. Oocytes injected with wild-type RNA were incubated with [^{14}C]mevalonate (50 μCi/mL) in the presence of 50 μM lovastatin. After 18 h, oocytes were transferred to fresh medium without label and lovastatin but supplemented with 2 mM cold mevalonolactone. After a 48-h chasing period, oocytes were manually dissected, and ^{14}C-labeled proteins were immunoprecipitated from membrane (M) and cytoplasm (C) fractions and analyzed by SDS–PAGE and fluorography. This was a 10-day exposure.

without lovastatin and label but supplemented with unlabeled 2 mM mevalonate and chased for 48 h. Figure 4 shows that the xlcaax-1 protein was indeed labeled and distributed between the cytosolic and membrane fractions, though there was more protein in the cytosolic fraction than in the membrane. We do not know why the distribution of the [^{14}C]mevalonate-labeled xlcaax was different than that of the [^{35}S]methionine-labeled protein; however, it may be due to a turnover of the prenyl groups on the xlcaax-1 protein associated with the membrane.

Mutation of the CAAX Motif Perturbs Xlcaax Localization. We have assumed, by analogy to the ras and ras-related proteins, that the prenylation of xlcaax-1 is signaled by its CAAX motif. Thus, the effect of mutating the CAAX motif on the membrane association of xlcaax-1 protein should be analogous to inhibiting isoprenylation. To test this, we substituted serine for Cys-588 (Figure 2, mutant Cys-588) in the CAAX motif of xlcaax-1 and analyzed the subcellular distribution of the mutant protein. Figure 2 shows that the ability of the mutated protein to associate with the oocyte plasma membrane was completely abolished. Densitometric measurements of the immunoprecipitated radioactive bands on an autoradiograph showed that 97% of the protein remained cytoplasmic and only 3% coisolated with the membrane fraction (Table I). We believe that the value of 3% of radioactive protein in the membrane represents contamination with cytosolic protein. We conclude that the mutation of the CAAX motif cysteine resulted in full abrogation of the membrane avidity of the xlcaax protein in the oocyte.

Since the blocking of xlcaax isoprenylation (by mutation in CAAX or by a prenylation inhibitor) should also prevent three subsequent CAAX-specified modifications (proteolytic removal of the last three amino acids, carboxymethylation of the C-terminal cysteine, and palmitoylation of upstream cysteines), the exact contribution of prenylation to the membrane binding of xlcaax is difficult to assess. Therefore, we analyzed the effect of mutations of Cys-585 and -587, which are potential palmitoylation sites of xlcaax-1, on the distribution of the protein in the oocyte.

Cysteine Residues 585 and 587 Upstream of the Caax Motif Are Both Required for Membrane Association. Our study of the posttranslational modifications of xlcaax-1 showed that this protein undergoes palmitoylation in XTC cells and when expressed in the baculovirus expression system (Kloc et al., 1991). Wild-type xlcaax-1 protein contains two cysteine residues, at positions 585 and 587 amino terminal to the CAAX motif, which are putative palmitoylation sites. We have produced constructs that mutate cysteines-585 and -587 individually to serine and have also made a double mutant converting both cysteines to serines. RNAs made from mutant templates were injected into the oocytes. After overnight incubation, we assayed the cytosolic and membrane distribution of the [^{35}S]methionine-labeled protein (Figure 2 and Table I). Mutations of Cys-585 and Cys-587 individually substantially reduced the amount of protein associated with the membrane: 81–89% of the protein was cytosolic, and 11–19% remained bound to the membrane (Figure 2). The double mutant (Cys-585 and -587) also reduced (to 16%) the amount of xlcaax-1 protein coisolating with the oocyte plasma membrane (Figure 2 and Table I). These results suggested that both cysteine palmitoylation sites are equally important for the strong association of xlcaax-1 with the plasma membrane and that the presence of the CAAX motif plus polybasic domain is a weak signal for membrane association.

The Polybasic Domain Is Not Necessary for Xlcaax-1 Association with the Oocyte Membrane. Some of the proteins belonging to the ras or ras-related family do not have the cysteine palmitoylation sites upstream of the CAAX motif but instead contain a stretch of positively charged lysine and/or arginine residues in close vicinity of the CAAX box [see Table I in Kloc et al. (1991)]. Hancock et al. (1990) demonstrated that the stretch of lysines in Kras4B serves a function analogous to the palmitoylation. Substitution of glutamines for lysines caused the protein to be cytosolic (Hancock et al., 1990). It is very intriguing that xlcaax-1, unlike most other CAAX proteins, contains two cysteine palmitoylation sites and a five amino acid long polybasic domain that resemble two redundant membrane association signals. Since we had already demonstrated (see above) that both of those two cysteines have a crucial role in the binding of xlcaax to the membrane, we defined the role of the polybasic domain in the plasma membrane association of xlcaax. Figure 2 and Table I show that the deletion of the polybasic domain while the CAAX motif and upstream cysteines were kept intact did not affect xlcaax-1 localization in the oocyte: it remained comparable to membrane association of the wild-type protein. This suggests that the polybasic domain does not play a major role in the membrane association of xlcaax-1 in oocytes.

Xlcaax-1 Is an ATP Binding Protein. The Xlcaax-1 protein contains an ATP binding consensus sequence that is shared by many ATP binding proteins (Miller et al., 1989). To confirm our earlier suggestion (Kloc et al., 1991) that xlcaax-1 is an ATP binding protein, we analyzed the ATP binding ability of the xlcaax-1 protein translated in *Xenopus* oocytes. *In vitro* synthesized xlcaax-1 mRNA was injected into *Xenopus* oocytes. After an overnight incubation at 18 °C, the translated proteins were immunoprecipitated using T7 tag antibody, separated on SDS–PAGE, and blotted onto nitrocellulose. Blots were incubated with labeled ATP or GTP in the presence or absence of the competitor (unlabeled ATP or GTP). In some experiments, we coinjected xlcaax-1 RNA with xnf7 RNA (does not bind ATP), and after overnight incubation, xlcaax-1 and xnf7 proteins were coimmunoprecipitated using

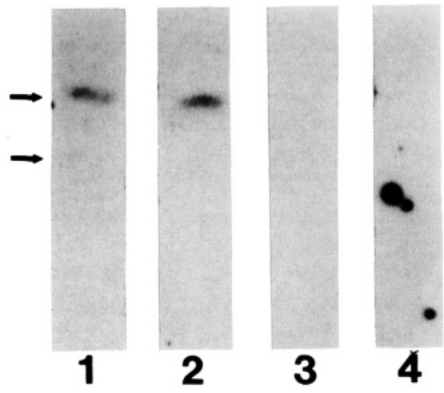

FIGURE 5: Xlcaax is an ATP binding protein. Oocytes were injected with *in vitro* synthesized wild-type xlcaax or coinjected with wild-type xlcaax and xnf7 RNA. After 18-h incubation, proteins were immunoprecipitated with the T7 antibody and separated on SDS–7.5% PAGE. After being transferred to nitrocellulose, the nucleotide binding assays were performed as described under Materials and Methods. Lane 1, xlcaax coinjected with xnf7; the upper arrow marks the position of xlcaax protein bound to ^{32}P-labeled ATP while the lower arrow marks the position of the unlabeled xnf7 protein band. Lane 2, xlcaax protein alone, [^{32}P]ATP-labeled band. Lane 3, competition of labeled ATP binding to xlcaax by preincubation with 1 μM cold ATP. Lane 4, incubation of xlcaax with [^{32}P]GTP.

T7 tag antibody. Figure 5 shows that the xlcaax-1 protein bound ATP but not GTP under the same experimental conditions. The binding of labeled ATP could be prevented by preincubation of the blot with 1 μM unlabeled ATP. In contrast, the xnf7 protein did not bind ATP or GTP under the same conditions (Figure 5).

DISCUSSION

We have found that xlcaax-1 protein translated in *Xenopus* oocytes from the injected *in vitro* synthesized RNA template is predominantly membrane-bound which is similar to the localization pattern of endogenous xlcaax-1 in *Xenopus* embryonic tissue culture cells (XTC cells) and in oocytes (Kloc et al., 1991). The association of xlcaax-1 with the oocyte plasma membrane is prenylation-dependent, and the protein becomes cytosolic when isoprenylation is blocked by an inhibitor or by mutation of the CAAX box cysteine to serine (mutant Cys-588).

The critical contribution of CAAX motif-signaled modifications to membrane targeting of the CAAX box containing proteins has been demonstrated for different ras proteins and nuclear lamins. For example, mutant ras proteins lacking either the CAAX cysteine or the AAX residues are not prenylated or palmitoylated and remain cytosolic (Hancock et al., 1989; Kato et al., 1992). Similarly, mutation of the cysteine residue of the CAAX box or introduction of a stop codon immediately after the cysteine residue impairs the association of lamin B2 with the inner nuclear membrane (Kitten & Nigg, 1991). The results of the present study also show that mutation of the CAAX cysteine of xlcaax-1 abolishes membrane association of the protein in oocytes.

We also show that prenylation alone of xlcaax-1 does not confer a strong membrane affinity since the mutated forms of xlcaax with an intact CAAX domain but a mutation in one or both upstream cysteine palmitoylation sites (mutants Cys-585, Cys-587, and Cys-585 and -587) remained predominantly cytosolic. This indicates that although the CAAX-signaled modifications are necessary for membrane localization of xlcaax-1, the two cysteine palmitoylation sites upstream of the CAAX motif provide a secondary membrane-targeting signal that promotes strong membrane binding.

The results of several studies on CAAX-containing proteins also showed that the CAAX motif by itself does not confer optimal membrane affinity. Mutational analysis demonstrated that the CAAX motif alone is insufficient for plasma membrane localization of pH21 ras, pN ras, and pK rasA and that a combination of the CAAX motif with the upstream cysteine palmitoylation sites is necessary to target p21 ras to the plasma membrane (Hancock et al., 1990). It has been shown that the addition of ras CAAX sequences to the carboxy terminus of heterologous protein A promotes its isoprenylation; however, it is not sufficient to localize the chimeric protein to the membrane (Hancock et al., 1989). The addition of the whole C-terminal domain containing the CAAX box and upstream cysteine palmitoylation sites, however, efficiently targets protein A to the plasma membrane (Hancock et al., 1991).

The p21H ras contains two cysteine palmitoylation sites at positions 181 and 184, upstream of the CAAX motif. Hancock et al. (1990) showed that the wild-type p21 ras with two palmitoylation sites intact and a mutant with a single palmitoylation site (Cys-184) are both associated with the plasma membrane. This demonstrates that in p21H ras a single palmitoylation site is enough to complement the CAAX motif and efficiently target the protein to the membrane. The xlcaax-1 protein also has two cysteine palmitoylation sites upstream of the CAAX motif. However, our present study showed that in xlcaax-1 both cysteines are necessary for tight protein binding to the oocyte plasma membrane. Mutants with only a single putative palmitoylation site (Cys-585 or Cys-587 mutated to Ser) did not associate efficiently with the plasma membrane and instead remained predominantly cytosolic, as did the double mutant with no palmitoylation sites (both Cys-585 and -587 mutated). This suggests that xlcaax-1, being a large hydrophilic molecule compared to other members of the family, may require two palmitate groups which increase hydrophobicity to associate with the oocyte plasma membrane. Alternatively, the mutation of a single cysteine in xlcaax-1 could cause a change in the conformation of the protein that hinders the palmitoylation of the second cysteine, resulting in the single cysteine mutant of xlcaax-1 behaving like a double mutant. We also repeated this experiment using the full-length xlcaax-1 protein and found that protein with mutated Cys-587 did not associate efficiently with the membrane, indicating that the truncated xlcaax construct used in this experiment behaved as the full-length protein (data not shown). Recently, Adamson et al. (1992) showed that both cysteine residues (188 and 192) serve as sites for palmitoylation in p21^{rhoB}. Interestingly, mutations in Cys-192 resulted in the incorporation of C15 farnesyl instead of C20 geranylgeranyl groups on the CAAX box cysteine, indicating an interaction between the palmitoylation signals and the CAAX box cysteine.

Several proteins from the ras family have a polybasic amino acid domain immediately upstream of the carboxy-terminal CAAX motif [see Table I in Kloc et al. (1991)]. Characteristically, all these proteins lack the cysteine palmitoylation sites present in Nras, Hras, and K-ras4A. The results of mutational analyses indicate that the lysine residues of the polybasic domain in K-ras4B serve a function analogous to that of palmitoylation. For example, replacement of the positively charged lysines with neutral glutamines causes K-ras4B to become cytosolic (Hancock et al., 1990). On the other hand, when the positively charged lysine residues were substituted with arginines, Kras4B was rendered membrane-bound (Hancock et al., 1991). Xlcaax-1 contains two cysteine

palmitoylation sites in addition to the arginine/lysine-rich polybasic domain. Our mutational analysis demonstrated that the two cysteines are essential for membrane localization but that the polybasic domain is dispensable for membrane association. These results suggest either that the polybasic domain is not important in membrane association of xlcaax-1 or that it is not functional in *Xenopus* oocytes and is functional in somatic cells.

The conceptual amino acid analysis of the xlcaax-1 protein indicated the presence of a potential nucleotide binding site (Miller et al., 1989). We were able to demonstrate the specific binding of ATP but not GTP to xlcaax-1 protein produced in oocytes. This strongly supports the hypothesis that xlcaax-1 function in epithelial cells *in vivo* involves association with the basal-lateral aspects of the plasma membrane and utilization of ATP. On the basis of its localization to the basal-lateral membrane in several polarized epithelia, it is likely that it is involved in ion transport (Cornish et al., 1992). In oocytes, however, xlcaax is distributed evenly to all membrane aspects (Kloc and Etkin, unpublished results). Therefore, an important question is the role of the various motifs and modifications in different patterns of localization between polarized epithelia and germ cells.

ACKNOWLEDGMENT

We thank members of the Etkin laboratory for their critical comments and discussions regarding the manuscript.

REFERENCES

Adamson, P., Marshall, C. J., Hall, A., & Tilbrook, P. A. (1992) *J. Biol. Chem. 267*, 20033–20038.

Cornish, J. A., Kloc, M., Decker, G. L., Reddy, B. A., & Etkin, L. D. (1992) *Dev. Biol. 150*, 108–120.

Etkin, L. D., & Maxson, R. E. (1980) *Dev. Biol. 75*, 13–25.

Fujiyama, A., & Tamanoi, F. (1990) *J. Biol. Chem. 265*, 3362–3368.

Gutierrez, L., Magee, A. I., Marshall, C. J., & Hancock, J. F. (1989) *EMBO J. 8*, 1093–1098.

Hancock, J. F., Magee, A. I., Childs, J. E., & Marshall, C. J. (1989) *Cell 57*, 1167–1177.

Hancock, J. F., Paterson, H., & Marshall, C. J. (1990) *Cell 63*, 133–139.

Hancock, J. F., Cadwallader, K., & Marshall, C. J. (1991) *EMBO J. 10*, 641–646.

Hurley, J. B., Fong, H. K., Teplow, D. B., Dreyer, W. J., & Simon, M. I. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6984.

Jackson, J. H., Cochrane, C. G., Bourne, J. R., Solski, P. A., Buss, J. E., & Der, C. J. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3042–3046.

Kato, K., Cox, A. D., Hisaka, M. M., Graham, S. M., Buss, J. E., & Der, C. J. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6403–6407.

Khosravi-Far, R., Cox, A. D., Kato, K., & Der, C. J. (1992) *Cell Growth Differ. 3*, 461–469.

Kitten, G. T., & Nigg, E. A. (1991) *J. Cell Biol. 113*, 13–23.

Kloc, M., Reddy, B., Crawford, S., & Etkin, L. D. (1991) *J. Biol. Chem. 266*, 8206–8212.

Lapetina, E. G., & Reep, B. R. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 2261–2265.

Maltese, W. A., & Sheridan, K. M. (1990) *J. Biol. Chem. 265*, 17883–17890.

Marcus, S., Caldwell, G. A., Miller, D., Xue, C.-B., Naider, F., & Becker, J. M. (1991) *Mol. Cell. Biol. 11*, 3603–3612.

Miller, M., Kloc, M., Reddy, B., Eastman, E., Dreyer, C., & Etkin, L. D. (1989) *Genes Dev. 3*, 572–583.

Nigg, E. A., Kitten, G. T., & Vorburger, K. (1992) *Biochem. Soc. Trans. 20*, 500–504.

Reddy, B. A., Kloc, M., & Etkin, L. D. (1991) *Dev. Biol. 148*, 107–116.

Sadler, S. E., & Maller, J. L. (1981) *J. Biol. Chem. 256*, 6368–6373.

Vorburger, K., Kitten, G. T., & Nigg, E. A. (1989) *EMBO J. 8*, 4007–4013.