Isolation of the Human γ-Carboxylase and a γ-Carboxylase-Associated Protein from Factor IX-Expressing Mammalian Cells

Susan E. Lingenfelter[‡] and Kathleen L. Berkner*[,§]

ZymoGenetics, Inc., Seattle, Washington 98102, and Department of Cell Biology, Cleveland Clinic Foundation, Cleveland, Ohio 44195

Received September 29, 1995; Revised Manuscript Received April 10, 1996[⊗]

ABSTRACT: A model system for the analysis of intracellular events governing the modification of individual vitamin K-dependent (VKD) proteins by the carboxylase has been developed using recombinant VKD protein-transfected cell lines. When untransfected 293 cells were analyzed by *in vitro* carboxylation followed by SDS−PAGE, endogenous VKD proteins were not detected. With 293 cells stably-transfected with recombinant native factor IX, most (>95%) of the carboxylase was in complex with the factor IX, as assayed by adsorption of carboxylase activity to immobilized anti-factor IX antibody. In contrast, with 293 cells stably-transfected with recombinant factor IX deleted in the propeptide sequence (amino acids −18 to −4, Δpro factor IX), no association of factor IX with the carboxylase was observed. This observation was used to specifically isolate and identify the human carboxylase, and a carboxylase-associated protein. When the carboxylase was purified from solubilized microsomes from either native factor IX, or Δpro factor IX, stably-transfected 293 cells, a single 98 kDa band was specifically obtained from native factor IX microsomes, but not from Δpro factor IX microsomes. This band was subsequently shown by Western and microsequencing analysis to comprise both the carboxylase and a carboxylase-associated protein. This isolation, which represents the first isolation to near homogeneity of both the human carboxylase and the carboxylase from cell lines, will be valuable in isolating enzymatically active recombinant carboxylase, which has been refractile to other purification attempts. This system was also used to show that the human carboxylase in 293 cells is capable of binding and modifying two different liver-derived proteins. Protein C-producing 293 cells were generated from the same 293 progenitor cell line used to create the factor IX-expressing cells. With both factor IX- and protein C-transfected 293 cells, the secreted proteins were almost completely carboxylated, and in microsomes from each cell line the carboxylase was found in near quantitative complex with the two different VKD proteins. Thus the carboxylase modifies both VKD proteins. The approach described here for the analysis of the carboxylase from recombinant VKD protein-transfected cell lines should provide an important new system for studying protein carboxylation and VKD protein−carboxylase interaction.

The microsomal VKD[1] carboxylase catalyzes the conversion of glutamic acids to γ-carboxylated glutamic acids, or glas, in a reaction that requires O_2, CO_2, and vitamin K hydroquinone (Suttie, 1985; Friedman & Przysiecki, 1987; Furie & Furie, 1990; Vermeer, 1990). The substrates for the carboxylase, the VKD proteins, are carboxylated at a cluster (3−12) of glutamic acids in a region of the protein termed the gla domain. Carboxylation of these proteins is required for their interaction with phospholipid bilayers and for their biological activity. Approximately one dozen VKD proteins have been identified, including several blood proteins (prothrombin, protein C, protein Z, protein S, factor IX, factor VII, and factor X), the bone gla protein and a protein synthesized in multiple tissues, the matrix gla protein (Fraser & Price, 1988; Furie & Furie, 1988; Price, 1988), and a protein involved in growth specific arrest (Manfioletti et al., 1993; Varnum et al., 1995). The cDNAs encoding these VKD proteins have been isolated, and most have been expressed in mammalian cells. The production and secretion of recombinant VKD proteins that resemble the natural source of protein is complicated by the limited carboxylation capacity observed in mammalian cell systems. At low levels of VKD protein production, the protein is secreted in an accurately post-translationally modified form. However, as production levels increase, carboxylation is impaired, and only a subpopulation of fully carboxylated protein is obtained. Efficiency of secretion of carboxylated protein varies among cell lines, with the 293 cell line, a transformed human embryonic kidney line, being one of the most efficient for secretion of active VKD proteins (Berkner et al., 1986; Grinnell et al., 1987).

All VKD proteins have in common an approximate 18 amino acid sequence unique to this subset of proteins. In most cases, this sequence comprises a propeptide located N-terminal to the gla domain, which is removed during the secretion of these proteins. An exception to this organization is the matrix gla protein, where this peptide is embedded within the mature protein, and where the gla domain straddles the peptide sequence (Price, 1988). Such structural diversity raises the question whether there are multiple carboxylases;

* To whom correspondence should be addressed.
‡ ZymoGenetics, Inc.
§ Cleveland Clinic Foundation.
⊗ Abstract published in *Advance ACS Abstracts,* June 1, 1996.
[1] Abbreviations: CHAPS, 3-[3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Δpro factor IX, propeptide-deleted factor IX; VKD, vitamin K dependent; PMSF, phenylmethanesulfonyl fluoride; GCAP, γ-carboxylase-associated protein.

S0006-2960(95)02331-2 CCC: $12.00

however, it has not yet been determined whether a single isoform can modify different VKD proteins. The propeptide sequence is important for carboxylase recognition (Foster et al., 1987; Jorgensen et al., 1987) and may also be involved in regulation of the reaction (Knobloch & Suttie, 1987). Identification of the propeptide as a carboxylase recognition sequence has been critical in the purification of this enzyme. An apparently homogeneous preparation of bovine carboxylase was obtained using the propeptide to displace the carboxylase from a carboxylase−prothrombin complex initially bound to immobilized anti-prothrombin antibody (Berkner et al., 1992). The propeptide (Hubbard et al., 1989), or the propeptide plus gla domain (Wu et al., 1991b), has also been used directly as an affinity ligand to purify the bovine carboxylase. In the latter case, a near-homogeneous protein preparation was obtained. Carboxylase-encoding bovine and human cDNAs have been isolated, using amino acid sequence derived from purified bovine liver protein (Wu et al., 1991a). The bovine cDNA has been identified as encoding the carboxylase by its ability to effect carboxylase activity in baculovirus-infected insect cells, which do not otherwise contain endogenous activity (Roth et al., 1993). The carboxylase activity measured was on a synthetic pentapeptide substrate. In contrast, when the bovine carboxylase cDNA was transfected into CHO cells expressing undercarboxylated factor IX, the cDNA did not affect protein carboxylation (Rehemtulla et al., 1993).

Despite the fact that several VKD proteins have been expressed in a variety of mammalian cells, the analysis of carboxylation of these proteins has been limited to indirect methods characterizing the secreted proteins. Recombinant VKD protein-expressing cell lines provide several potential advantages over tissue for the direct analysis of the VKD protein−carboxylase interaction, including the ability to study the interaction of individual VKD proteins with the carboxylase as well as the use of mutated recombinant forms. For this reason, we have developed a model system, using cell lines uniquely expressing individual VKD proteins, to directly analyze the intracellular events in VKD protein carboxylation. Using this system, we show that a single carboxylase is capable of modifying two different VKD proteins (factor IX and protein C). In addition, the human carboxylase and a carboxylase-associated protein have been purified from 293 cell lines stably-transfected with recombinant factor IX. Both proteins have been shown to specifically interact with the factor IX precursor by their requirement for the factor IX propeptide for binding. The approach described here for the isolation and analysis of the carboxylase from recombinant VKD protein transfected cell lines should provide a framework for future studies on the recombinant carboxylase, which to date has been refractile to purification (Kuliopulos et al., 1994).

MATERIALS AND METHODS

Construction of Propeptide-Deleted Factor IX. A *Bam*HI fragment (1.4 kb) encoding a full-length factor IX cDNA was subcloned into pUC 118, oriented so that the *Hin*dIII site in the pUC 118 polylinker was positioned at the 3′ end of the factor IX cDNA. Two fragments were isolated from this plasmid: an *Eco*RV−*Hin*dIII 3.2 kb fragment containing almost all of pUC 118 and the 5′ terminus (0−65 bp) of factor IX, and a 1.3 kb *Hae*III−*Hin*dIII fragment containing factor IX from 130 to 1390 bps. These fragments were ligated together with an oligonucleotide pair (5′-ATCTACT-CAGTGCTGAATGT and 5′-ACATTCAGCACTGAGTA-GAT), generating a plasmid encoding factor IX deleted in 15 amino acids in the propeptide (i.e., amino acids −18 through −4). DNA manipulations were performed according to established procedures (Sambrook et al., 1989). The 5′ terminus of factor IX (approximately 400 bps) was sequenced to determine that the change introduced was the correct, in-frame, deletion. The *Bam*HI fragment containing the Δpro factor IX cDNA was then subcloned into the mammalian expression vector pD5 (Berkner et al., 1988) and was subsequently cotransfected into 293 cells with pD5-Neo, an expression plasmid encoding a phosphotransferase gene conferring resistance to G418. A parallel cotransfection was performed with the pD5 expression vector containing native factor IX-encoding cDNA. The cells were selected in Dulbecco's DMEM media containing 10% dialyzed fetal calf serum, antibiotics, and G418 sulfate (GIBCO BRL), and 293 colonies expressing factor IX were screened by ELISA (described below) after clonal isolation. Approximately 2 dozen colonies were analyzed for each factor IX protein, and clones expressing comparable levels of intracellular factor IX were chosen for subsequent study.

Antibody Production. Polyclonal rabbit antisera to purified human plasma factor IX (Enzyme Research Laboratories, Inc.) were affinity purified on a column containing factor IX immobilized to Sepharose (10 mL, 1 mg/mL) and then coupled to CNBr-activated Sepharose (Pharmacia), at 5 mg/mL. A monoclonal antibody to the heavy chain of protein C, PCH-1, has been described previously (Foster et al., 1990). This antibody was purified on a protein A−Sepharose column (Sigma) and then coupled to CNBr-activated Sepharose at 5 mg/mL. Antibodies were also generated against two peptides: pepC1 (SSLDRKYLDGL) which is derived from the carboxylase sequence (Wu et al., 1991a) and pep G1 (RERFEKTFQLKEK) which is derived from GCAP, the human homologue of a protein purified from bovine liver (Berkner et al., 1992; Berkner et al., unpublished data). Both peptides were synthesized with an Applied Biosystems Model 431A peptide synthesizer and purified on a preparative C18 column (Vydac). Peptide sequences were verified by mass spectral analysis, and each peptide was then coupled to KLH and injected into rabbits. Antipeptide antisera were affinity purified on columns containing peptides immobilized to Sepharose (2 mg of peptide/mL). The antisera were tested for cross-reactivity between GCAP and carboxylase using ^{35}S-labeled *in vitro* translated GCAP or carboxylase. The details for subcloning of GCAP- or carboxylase-cDNAs into an *in vitro* expression vector and the conditions for *in vitro* transcription and translation will be presented elsewhere. Following the *in vitro* translation of each protein in rabbit reticulocyte lysates containing canine microsomes (50 μL), the samples were diluted 10-fold into 1% CHAPS and adsorbed to either 100 μL of anti-peptide C1 or anti-peptide G1 immobilized to Sepharose (at 5 mg/mL). After washing with 50 mM Tris, pH 7.4, 0.5 M NaCl, and 1% CHAPS, the resins were incubated in SDS−PAGE loading buffer and eluted proteins were then gel electrophoresed. As shown in Figure 1, the anti-peptide antisera were specific for GCAP or for carboxylase.

Purification of the Human Carboxylase. Confluent 293 cells (4×10^9, untransfected or stably-transfected with native factor IX or with Δpro factor IX) were dislodged from tissue

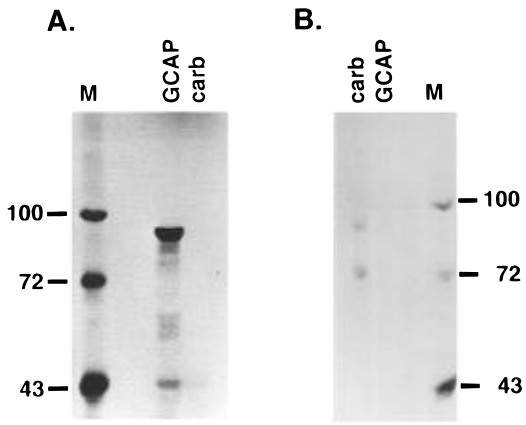

FIGURE 1: Anti-peptide reactivity with *in vitro* translated GCAP or carboxylase. ^{35}S-Labeled GCAP or carboxylase synthesized in a reticulocyte cell lysate with canine microsomes was tested for immunoreactivity with anti-peptide antisera to (A) GCAP (anti-peptide G1) or (B) carboxylase (anti-peptide C1), as described in the Materials and Methods. The two molecular weight carboxylase forms are due to glycosylation differences. Only the lower, unglycosylated, form was observed after *in vitro* translation in the absence of canine microsomes (unpublished data).

culture plates using Versene (GIBCO BRL). The cells were pelleted at 2000 rpm for 5 min, and then washed twice in 200 mL of phosphate buffered saline (PBS, GIBCO BRL), and recentrifuged. All procedures were performed at 4 °C, unless otherwise indicated. The cells were resuspended in 25 mL of buffer A (0.25 M sucrose, 0.025 M imidazole, pH 7.3, and 1 mM PMSF) and sonicated on ice using 4−15 s bursts, with 30 s intervals between each sonication. Vital dye exclusion, using Trypan blue (0.4%, GIBCO BRL), indicated greater than 99% cell breakage. The preparation was dounce homogenized and then centrifuged for 15 min at 4000 rpm. The postnuclear fraction was spun at 45 000 rpm for 1 h, the supernatant was removed, and the microsomal pellets were frozen in liquid N_2 and stored at −80 °C.

Microsomes were dounce homogenized in 25 mL of buffer B (50 mM Tris, pH 7.4, 100 mM NaCl, and 1 mM PMSF), then adjusted to 0.1% CHAPS, and rocked for 1 h. The preparation was centrifuged at 100000*g* for 1 h, and the pellet was subsequently homogenized in buffer B adjusted to 200 mM NaCl. CHAPS was added to a final concentration of 0.5%, and the sample was rocked for 1 h and then pelleted for 1 h at 100000*g*. To determine the recoveries of carboxylase and factor IX, carboxylase assays and factor IX ELISAs were performed on the 0.1% suspension, the 0.1% supernatant, the 0.5% suspension, and the 0.5% supernatant. The 0.5% supernatant was applied to an affinity purified anti-factor IX polyclonal antibody column (5 mL), and the column was rocked overnight. In a preliminary experiment, this 0.5% supernatant was first sonicated (100 × 2 s bursts) prior to administration to the column. However, the ultimate purity and recovery of carboxylase was unaffected by this manipulation, so this procedure was discontinued. Following the overnight incubation, the flow-through was collected and the column was rinsed with 100 mL of buffer C (50 mM Tris, pH 7.4, 500 mM NaCl, 0.25% CHAPS (Pierce), 0.25% L-α-phosphatidylcholine IIIE (Sigma), 5 mM DTT) and then with 25 mL of buffer C containing 1 mM ATP (Sigma) and 5 mM MgCl. This final wash removed BiP (Munro & Pelham, 1986), a major contaminant in the preparation. All washes were performed at 20 °C. In trial experiments, a linear gradient of Triton X100 (0.1−0.5%, Pierce) in buffer C (50 mL total) was also included, as the final column wash. As this detergent was found to have no effect on subsequent propeptide eluant purity, its use was discontinued. The carboxylase was then eluted using buffer C containing an 18 amino acid peptide corresponding to the factor X propeptide (synthesized and analyzed by mass spectroscopy as for the peptides described above). The column was rocked with 5 mL of 100 μM propeptide in buffer C overnight at 20 °C, the eluant was collected, and the column was incubated for an additional 24 h in 5 mL more elution buffer. To monitor carboxylase adsorption to the antifactor IX column, the 0.5% supernatant, the supernatant plus resin, the washed resin, and the flow-through were assayed for carboxylase activity. To determine the recovery of carboxylase activity eluted from the anti-factor IX antibody column, the propeptide eluant and resin pre- and post-elution were also assayed. Adsorption and elution were near-quantitative (>90%) in both instances.

Isolation of Carboxylase−Protein C Complex. 293 cells (2×10^9) expressing native protein C or propeptide-deleted protein C [generously provided by Don Foster (Foster et al., 1987)] were used to prepare microsomes, exactly as described above. The microsomes were resuspended in 15 mL of buffer B adjusted to 0.2 M NaCl, dounce homogenized, and then solubilized in 0.5% CHAPS by rocking the samples for 1 h at 4 °C and centrifuging at 100000*g* for 1 h. Almost all of the carboxylase activity was solubilized under these conditions as determined by measuring carboxylase activity in the 0.5% suspension and 0.5% supernatant. The solubilized material was incubated overnight at 4 °C with anti-protein C antibody (5 mL of PCH-1−Sepharose), and the column was then washed with buffer C. Solubilized microsomes, column flow-through, and resin containing the protein C−carboxylase complex were then quantitated for activity using the carboxylase assay.

Protein Analysis. Factor IX levels were determined by ELISA, using a cocktail of monoclonal antibodies (ESN2 and ESN4, American Diagnostica, Inc.) as the capturing antibodies, and affinity purified polyclonal antifactor IX antibody for antigen detection. Both uncarboxylated and carboxylated factor IX molecules were recognized equally well using this assay (data not shown). The carboxylase assay was performed as described previously (Berkner et al., 1992), except for an increase in peptide concentration (3 mM Boc-Glu-Glu-Leu-OMe or EEL, Bachem) and for the addition here of 50 mM BES, pH 7.2, and 20 μM factor X propeptide. *In vitro* carboxylation of intracellular factor IX was analyzed by incubating 50 μg of 0.5% supernatant with 50 μL of the carboxylase reaction cocktail, followed by boiling the samples in SDS−PAGE loading dye, gel electrophoresis, and fluorography. Protein concentration was determined by BCA (Pierce) analysis.

Western analysis of the propeptide eluant was performed using affinity purified antipeptide antibodies at concentrations of 2−5 μg/mL and iodinated protein A [2×10^6 dpm/μg, (Berkner et al., 1992)]. Microsequence analysis of the propeptide eluant from native factor IX or Δpro factor IX microsomes (isolated from 10^{10} or 4×10^9 cells, respectively) was performed after concentration of the propeptide eluant by Centricon-30 ultrafiltration (Amicon). The samples were gel electrophoresed and transferred to nitrocellulose as described (Aebersold et al., 1987) but with 0.005% SDS in the transfer buffer. The nitrocellulose was stained using

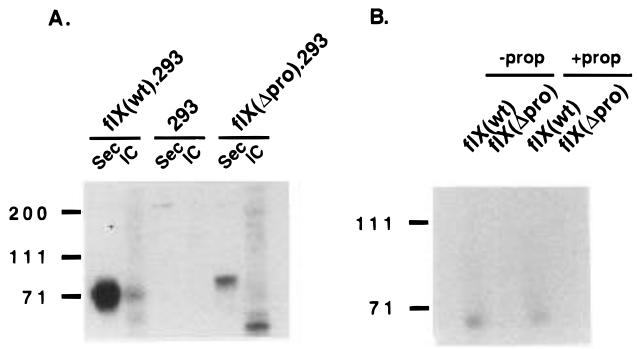

FIGURE 2: *In vivo* labeling and *in vitro* carboxylation of factor IX from 293 cell lines. (A) Equal numbers of untransfected 293 cells or wild type (wt) or propeptide-deleted (Δpro) factor IX-producing 293 cells were labeled for 2 h *in vivo*, as previously described (Berkner, 1993), in media containing vitamin K. Protein secreted into the media (Sec) or intracellular protein (IC) solubilized using RIP-A buffer (Harlow & Lane, 1988) was then immunoprecipitated using an excess of affinity purified polyclonal α-factor IX antibody. The differences in molecular weight between the two secreted factor IXs are due to glycosylation differences. (B) Microsomes were prepared from cells cultured in vitamin K-depleted media. Solubilized microsomes (50 μg) were incubated in the carboxylase reaction (as described in the Materials and Methods but without the peptide substrate) in the presence or absence of 20 μM factor X propeptide (prop). Samples were then gel electrophoresed and processed for fluorography.

Amido Black (0.1% in water), then washed with water, and sent to the Harvard Microchemistry Facility for internal amino acid sequence determination.

Gla quantitation was determined using previously described methods (Berkner, 1993). Factor IX- or protein C-producing 293 cells were cultured for 3 days in serum free media (Berkner, 1993) containing 5 μg/mL vitamin K, and the proteins were then immunopurified using either an immobilized monoclonal antibody against the heavy chain of protein C (PCH-1) or an immobilized monoclonal antibody against factor IX (ESN 1, Diagnostica, Inc., coupled to Sepharose at 2 mg/mL). The ESN 1 antibody recognized both carboxylated and noncarboxylated factor IX.

RESULTS

Generation and Characterization of Native or Δpro Factor IX-Expressing 293 Cells. A Δpro factor IX cDNA that differed from native factor IX by the in-frame deletion of amino acids −18 to −4 in the propeptide was generated, as described in the Materials and Methods. Stably-transfected 293 cell lines were generated for both the Δpro factor IX-encoding cDNA and for the native factor IX cDNA. When multiple transfectants secreting either factor IX form were analyzed for intracellular and secreted levels of factor IX by ELISA or by *in vivo* labeling and immunoprecipitation, impaired secretion of the Δpro factor IX was observed (shown for representative isolates in Figure 2A). The level of Δpro factor IX secretion was also much lower than observed for native factor IX secreted from vitamin K-depleted 293 cells (data not shown). Impaired secretion was thus not due to the fact that the Δpro factor IX could not be carboxylated, but was more likely due to improper folding of this protein due to the lack of the propeptide sequence. To analyze native and Δpro factor IX cell lines that expressed comparable levels of factor IX, cell lines with similar intracellular levels of factor IX were chosen (Table 1).

Table 1: Fractionation of Factor IX in the Carboxylase Purification[a]

fraction	native factor IX (μg)	propeptide-deleted factor IX (μg)
0.1% suspension	185	165
0.1% supernatant	72	81
0.5% suspension	113	77
0.5% supernatant	63	59
α-factor IX−Sepharose flow-through	<0.01	<0.01

[a] Samples were assayed for factor IX by ELISA, as described in the Materials and Methods. All aliquots were diluted into buffer containing 0.5% CHAPS to ensure total solubilization of the factor IX before assay. Factor IX adsorption to anti-factor IX resin was also shown by treating the resins, after carboxylase elution with propeptide, with 0.1 M sodium citrate, pH 2.5. ELISA analysis of the low pH eluant gave factor IX amounts similar to those present in the respective 0.5% supernatants shown above.

Deletions or mutations in the propeptide sequence have been shown to impair the carboxylation of secreted VKD proteins, and we observed this impaired carboxylation with intracellular factor IX, as well. While wild type factor IX carboxylation could be detected in an *in vitro* carboxylation reaction, no carboxylation of Δpro factor IX was observed (Figure 2B). Carboxylation of Δpro factor IX could not be stimulated *in trans* by an exogenously added propeptide. This analysis would also detect any endogenous VKD proteins in 293 cells; however, none were observed.

The specific activity for solubilized factor IX-containing 293 microsomes [85 cpm/(h·μg), Table 2A] was approximately one-half that of bovine liver microsomes, when directly compared (data not shown). We previously reported a specific activity of 87 cpm/(h·μg) for detergent-solubilized bovine liver microsomes, using an assay that was about 2-fold less sensitive than that used in these studies (Berkner et al., 1992). The level of carboxylase activity in untransfected 293 microsomes or in Δpro factor IX microsomes was approximately 2-fold lower than for native factor IX microsomes (54 and 50 cpm/(h·μg) Table 2A−C). This difference in carboxylase activities was observed for several independent cell lines expressing the two different factor IX forms. When these cell lines were quantitated for carboxylase using a carboxylase anti-peptide antibody, no differences in carboxylase levels were observed (Figure 3A). Carboxylase peptide activity could be stimulated by exogenously added propeptide. Stimulation (which was observed for fractions at all stages of purification and is represented in Table 2D by the 0.5% supernatant) was greater in untransfected 293 microsomes (12.5-fold) than in native factor IX 293 microsomes (1.9-fold).

Purification of the Human Carboxylase. Optimal conditions for the isolation of the carboxylase were initially determined using the native factor IX-producing 293 cell line, and the purification was then carried out in parallel with untransfected 293 cells and with Δpro factor IX-expressing 293 cells. The purification was from cells cultured in vitamin K-depleted media, to maximize the association of factor IX with the carboxylase (DeMetz et al., 1981). Supplementing vitamin K-containing media with warfarin gave essentially the same results (data not shown). In initial experiments, the NaCl and detergent (CHAPS) concentrations used in solubilizing the microsomes were varied, at a fixed protein concentration, to optimize for recovery and for ultimate purity of the propeptide eluant. Microsomes were solubilized

Table 2: Purification of the Carboxylase from 293 Cells[a]

(A) Native Factor IX-293 Cells

fraction	carboxylase act. (cpm/h)	protein (mg)	sp. act. (cpm/(h·μg))	fold purification
0.1% suspension	2.2×10^7	260	85	1
0.1% supernatant	1.2×10^6	120		
0.5% suspension	2.0×10^7	140		
0.5% supernatant	1.2×10^7	60	200	2.4
α-factor IX−Sepharose	1.0×10^7			
flow-through	4×10^5	60		
propeptide eluant	9.0×10^6	0.003	3×10^6	3.5×10^4

(B) Propeptide-Deleted Factor IX 293 Cells

fraction	carboxylase act. (cpm/h)	protein (mg)	sp act. [cpm/(h·μg)]
0.1% suspension	1.2×10^7	220	54
0.1% supernatant	8×10^5	80	
0.5% suspension	8.4×10^6	140	
0.5% supernatant	4.8×10^6	60	80
α-factor IX−Sepharose	0		
flow-through	4.8×10^6	60	80
propeptide eluant	0		

(C) Untransfected 293 Cells

fraction	carboxylase act. (cpm/h)	protein (mg)	sp act. [cpm/(h·μg)]
0.1% suspension	1.1×10^7	220	50
0.1% supernatant	8×10^5	100	
0.5% suspension	6.4×10^6	140	
0.5% supernatant	4.0×10^6	60	67
α-factor IX−Sepharose	0		
flow-through	4.0×10^6	60	67
propeptide eluant	0		

(D) Propeptide Activation

sample	−propeptide	+propeptide	fold activation
native factor IX 293	6.4×10^6	1.2×10^7	1.9
untransfected 293	3.2×10^5	4.0×10^6	12.5

[a] Microsomes from 293 cells transfected with native factor IX (A) or Δpro factor IX (B), or from untransfected 293 cells (C), were solubilized and fractionated on anti-factor IX−Sepharose, as described in the Materials and Methods. The protein concentration determinations for pre-column samples were done by BCA. The propeptide eluant protein concentration was determined by densitometry of gel-electrophoresed samples, using BSA as a standard, as previously described (Berkner et al., 1992). The samples in parts A−C were assayed in the presence of 20 μM propeptide. The fractions assayed in part D were the 0.5% supernatants.

in two steps using increasing concentrations of CHAPS. Sequential solubilization was useful for the removal of free factor IX (i.e., uncomplexed to the carboxylase), which reduced the amount of anti-factor IX Sepharose subsequently required for adsorption of the factor IX−carboxylase complex and lowered the nonspecific protein background. When microsomes were treated with 0.1% CHAPS and 0.1 M NaCl, approximately 40% of the factor IX was solubilized, in contrast to only 5% solubilization of the carboxylase activity (Tables 1 and 2, Figure 3B). When the 0.1% CHAPS pellet from native factor IX-derived microsomes was subsequently solubilized with 0.5% CHAPS and then adsorbed onto immobilized anti-factor IX antibody, carboxylase activity was bound to the resin (Table 2A). Activity binding was near quantitative (96%), indicating that most of the carboxylase was in a complex with the native factor IX. In contrast, no carboxylase activity binding to anti-factor IX Sepharose was observed using microsomes from either Δpro factor IX cells or from the untransfected 293 cells (Table 2B,C).

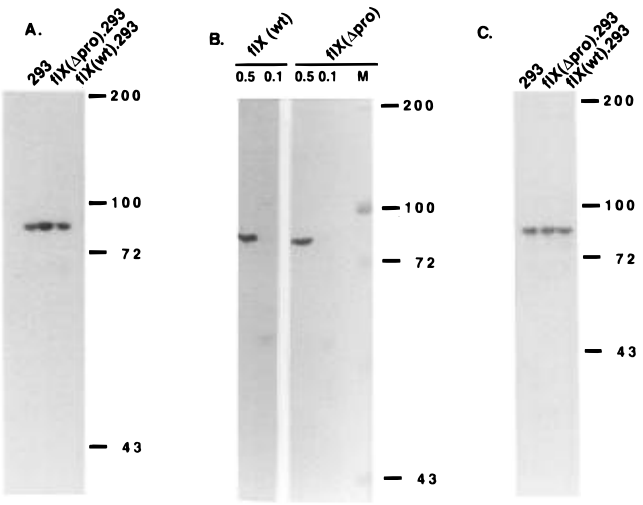

FIGURE 3: Western analysis of solubilized microsomes. Solubilized microsomes (supernatants from Table 2) were quantitated for protein by BCA analysis, and equal amounts of protein (100 μg) were gel electrophoresed, transferred to nitrocellulose, and probed with an affinity purified antibody to pepC1 (parts A and B) or to pepG1 (Berkner et al., 1992) (part C). The numbers above each lane in part B refer to percent CHAPS used to solubilize the microsomes. The samples in parts A and C were from 0.5% CHAPS solubilized microsomes.

Carboxylase elution from the antifactor IX resin was performed using a peptide comprising the factor X propeptide. Carboxylase activity elution was essentially quantitative (Table 2A), and this efficient recovery was also observed using a prothrombin propeptide or a factor IX propeptide, as well (data not shown). Optimal recovery required lengthy incubations with the propeptide: maximal recovery of activity, for example, took 2 days. The temperature (ambient) and high NaCl concentration (0.5 M) were both critical for optimal recoveries. When the elution was performed at 4 °C, for example, only 15% of the activity was eluted, over 2 days. The propeptide eluant specific activity was 3×10^6 cpm/(h·μg), representing a 3.5×10^4-fold purification over starting material (Table 2A). The difference in the amount of carboxylase (3 μg, Table 2) and factor IX (60 μg, Table 1 and Figure 4) recovered from the anti-factor IX resin showed that the intracellular levels of factor IX were in vast excess of the carboxylase.

When the propeptide eluants from native factor IX-, Δpro factor IX-, or untransfected-293 cells were analyzed by SDS−PAGE, a single 98 kDa band was observed, only in the native factor IX-derived propeptide eluant (Figure 5A). The purity of the preparation was dependent upon the concentration of CHAPS used in the sequential solubilizations and was also strongly dependent on the concentration of NaCl used in the column washes (0.5 M). When the NaCl concentration in the column wash buffer was reduced to 0.1 M, for example, three additional molecular weight forms were observed in the native factor IX propeptide eluant (Figure 5C). The purity of the propeptide eluant from Δpro factor IX microsomes was even more dramatically affected by changes in salt concentration. When low salt (0.1 M NaCl) buffer was used on the anti-factor IX column, several additional bands, including two abundant proteins with molecular weights of 110 and 55 kDa, were obtained in the propeptide eluant (Figure 5D). These two proteins were present in sufficient abundance to obtain sequence analysis of peptides derived from them, and they were identified as

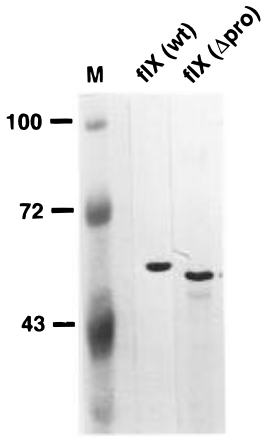

FIGURE 4: SDS−PAGE analysis of factor IX eluted from anti-factor IX resin. Factor IX was eluted from the antifactor IX resin, following the propeptide elution of carboxylase, as described in the legend to Table 1. An aliquot (10 μg, as determined by ELISA) was concentrated by Centricon filtration and subjected to SDS−PAGE and Coomassie staining. This isolation was performed from vitamin K-depleted cell lines. Only the lower molecular weight form of the doublet observed for intracellular factor IX isolated from vitamin K-containing cells (Figure 2) was detected under these conditions, as we have previously observed by *in vivo* labeling (Berkner, 1993).

a splicing factor, PSF (Gower et al., 1989; Patton et al., 1993), and NonO, an octamer binding protein (Dong et al., 1993; Yang et al., 1993).

The propeptide eluant derived from microsomes from tissue culture cells was substantially more pure than that obtained when bovine liver microsomes were used as the starting material. Figure 5B, for example, shows the propeptide eluant observed when prothrombin−carboxylase complex isolated from bovine liver was immunoadsorbed to immobilized anti-prothrombin antibody and then eluted with factor X propeptide. The purification of carboxylase from bovine liver or from 293 cells also differed in the effect of detergents in the column wash buffers. For example, Triton X100 was previously reported to increase the specific activity of the propeptide eluant derived from bovine liver microsomes (Wu et al., 1991b), and we observed this effect as well (Figure 5B). However, Triton X100 had no affect on the purity of the 293 cell line-derived material, even with propeptide eluant preparations substantially more impure than that shown in Figure 5A. Presumably, this difference in bovine liver tissue and in cultured cells is due to differences in their lipid content, and to the fact that 293 cells represent a homogeneous cell population compared to a complex tissue such as liver.

Native Factor IX Is Associated with Two Different Proteins. To identify the 98 kDa band observed in the propeptide eluant derived from native factor IX-expressing 293 cells, both Western and microsequence analyses were performed on the propeptide eluant. Two different bovine purifications to near or apparent homogeneity have been reported for the carboxylase (Wu et al., 1991b; Berkner et al., 1992). These purifications yielded two different proteins, as deduced from the cDNA sequences (Wu et al., 1991a; Berkner et al., unpublished data). Anti-peptide antisera specific to either the carboxylase or to the second protein, called GCAP (see Discussion) (Figure 1) were used to test for the presence of each protein in the propeptide eluant derived from factor IX-expressing 293 cells. Western analysis showed immunoreactivity of a 98 kDa band with antisera to each peptide (Figure 6). Thus, the apparent single molecular weight form observed for the factor IX 293 cell-derived propeptide eluant (Figure 5A) actually comprised two different proteins. Immunoreactivity was only observed with protein in the propeptide eluant from native factor IX-expressing 293 cells. No signal was detected in propeptide eluant from untransfected 293 cells (data not shown) or from Δpro factor IX-producing 293 cells (Figure 6). Thus, both proteins specifically associated with factor IX containing the propeptide.

Microsequence analysis confirmed the presence of two different proteins in the 98 kDa band of the propeptide eluant derived from native factor IX-expressing 293 cells. Sequences corresponding to peptides derived from both proteins were obtained from the 98 kDa band (Table 3). More peptides were obtained from the carboxylase sequence than from GCAP. However, the relative proportion of each protein in the 98 kDa band could not be determined, since this analysis is nonquantitative. This difference could reflect, for example, the ease of release of these peptides from nitrocellulose rather than the quantity of protein.

Western analysis of solubilized microsomes using antisera against a peptide derived from GCAP showed that this protein was present at equal levels in untransfected 293 cells and 293 cells containing either native or Δpro factor IX (Figure 3C). To test whether GCAP was the 98 kDa protein appearing in the propeptide eluants from resins washed using low salt conditions (Figure 5C,D), these propeptide eluants were analyzed by Westerns using the anti-peptide antisera. No immunoreactivity was observed (data not shown), indicating that these bands are not GCAP.

Two Different VKD Proteins Are Carboxylated by the Same Enzyme. The 293 cells provided a model system for testing whether the same endogenous carboxylase molecule can interact with more than one VKD protein. The observation that almost all (96%) of the carboxylase in 293 cells was in a complex with factor IX (Table 2A) was of interest because 293 cells have been shown to be efficient for the secretion of more than one carboxylated VKD protein. Fully active protein C (Foster et al., 1987; Grinnell et al., 1987) and factor IX (Berkner, 1993) have both been secreted from this cell line. Protein C, secreted from stably-transfected 293 cells generated from the same progenitor 293 cells used to express factor IX, was analyzed for gla content along with secreted factor IX (Table 4). Both proteins were extensively carboxylated, consistent with their observed full biological activities. The protein C-producing 293 cell line, and a propeptide-deleted protein C-expressing 293 cell line, were used to prepare microsomes, which were solubilized and then immunoadsorbed to immobilized anti-protein C antibody. When the resin and flow-through were assayed for carboxylase activity, near-quantitative adsorption of the carboxylase to the anti-protein C antibody was observed (Table 5). This interaction specifically depended upon the propeptide in protein C, since propeptide-deleted protein C did not capture carboxylase activity on the antibody resin (Table 5). Microsomes from the propeptide-deleted protein C cell line had about 3-fold lower carboxylase activity than those from the native protein C cell line (Table 5), similar to the results of the factor IX experiment (Table 2A,B). The fact that two different VKD proteins could quantitatively complex carboxylase activity in a propeptide-dependent manner

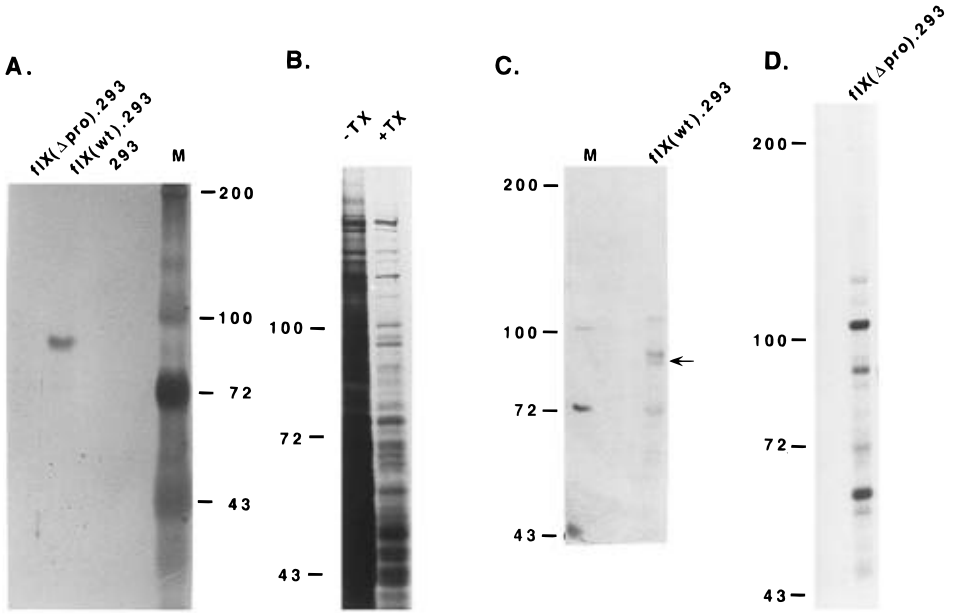

FIGURE 5: Gel electrophoretic analysis of purified carboxylase. Propeptide eluants from cell lines (A, C, D) or from bovine liver (B) were silver stained following SDS−PAGE. The propeptide eluants were obtained from bound α-factor IX (parts A, C, D) or α-bovine prothrombin (Berkner et al., 1992) (part B) antibody resins washed either with buffer C (parts A, B) or with a buffer identical to buffer C except for a reduced NaCl concentration (0.1 M) (parts C, D). In part B, the resin was washed in the presence or absence of a Triton X100 (TX) gradient, as described in the Materials and Methods. The arrow in part C indicates the same molecular weight form observed in part A.

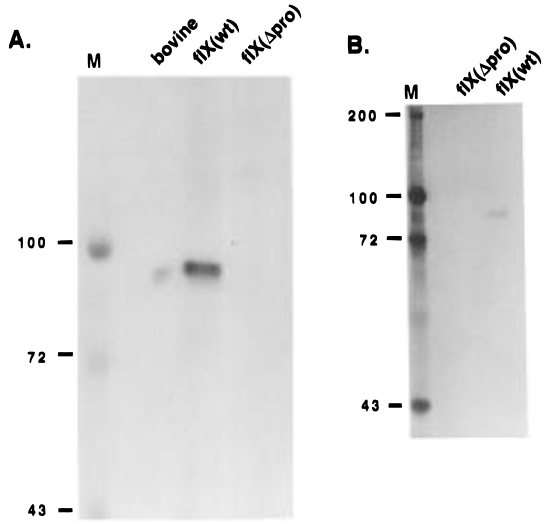

FIGURE 6: Western analysis of propeptide eluants. Propeptide eluants from native factor IX (7×10^5 cpm/h carboxylase activity), or from an equivalent volume of eluant from a Δpro factor IX preparation carried out in parallel, were concentrated 10-fold by Amicon ultrafiltration. Following gel electrophoresis, the proteins were transferred to nitrocellulose and probed using affinity purified antibodies to either pepG1 (A) or pepC1 (B). In part A, the S-Sepharose eluant from a bovine liver carboxylase preparation (Berkner et al., 1992) was also included as a control.

demonstrates that the same carboxylase modifies both proteins.

DISCUSSION

We have developed a novel system to study the intracellular interactions of VKD proteins with the carboxylase. The isolation of carboxylase−VKD protein complexes from cell lines expressing recombinant VKD proteins provides several advantages for the analysis of intracellular events as compared to whole tissues such as liver. The untransfected 293 cells were found to not contain detectable levels of endogenous VKD proteins (Figure 2B). Thus the interaction of the carboxylase with a single VKD recombinant protein introduced by transfection can be analyzed, in contrast to studies with the liver carboxylase, which is distributed among multiple VKD proteins. We observed that ~95% of the carboxylase in 293 cells was complexed with the single expressed VKD protein and used this information to then show that the same carboxylase that modifies factor IX also

Table 3: Identification of Peptides Isolated from the Propeptide Eluant 98 kDa Band[a]

peptide sequence	protein identity
L D A D (W) V E G Y S	carboxylase
G Q I F I V Y F I A G	carboxylase
G G P E P T P L V	carboxylase
T G E L G Y L N P	carboxylase
L G P L L D I L A	GCAP
F T L L P P T S P G	GCAP

[a] The 98 kDa band from Figure 5A was transferred to nitrocellulose and digested with trypsin, and the digest was chromatographed on a C18 column. Approximately 50 peaks were observed. When 6 individual peaks were subsequently analyzed by sequencing, each peak gave a unique sequence that could be unequivocally assigned to either the carboxylase or GCAP protein, as predicted by their respective cDNAs. A letter in parenthesis indicates a lowered confidence for identifying the amino acid.

Table 4: γ-Carboxyglutamic Acid Content of Factor IX and Protein C[a]

protein	gla (mol/mol of protein) expected	observed
human plasma factor IX	12	11.5 ± 0.2
native rec protein C	9	8.8 ± 0.2
native rec factor IX	12	10.5 ± 0.2
propeptide-deleted rec factor IX		<0.2

[a] Factor IX and protein C were purified on monoclonal antibody columns, as described in the Materials and Methods. Human plasma factor IX was purchased from Enzyme Research Laboratories, Inc. Purified recombinant (rec) proteins were shown to be homogeneous by Coomassie staining after SDS−PAGE. The gla content was determined as previously described (Berkner, 1993).

Table 5: Binding of Intracellular Protein C to the Carboxylase[a]

sample	carboxylase act. (cpm/h)	% act.
(A) Native Protein C		
solubilized microsomes	1.4×10^7	100
PCH-1 Sepharose	1.3×10^7	93
flow-through	5×10^5	4
(B) Propeptide-Deleted Protein C		
solubilized microsomes	5×10^6	100
PCH-1 Sepharose	2×10^4	<1
flow-through	5×10^6	100

[a] Solubilized microsomes from 293 cells expressing native protein C (A) or propeptide-deleted protein C (B) were adsorbed to PCH-1 Sepharose, as described in the Materials and Methods. The resin was washed with buffer C and assayed, along with starting material and flow-through, for carboxylase activity.

modifies protein C. We found that the same 293-derived carboxylase protein binds intracellular protein C or factor IX nearly quantitatively (Tables 2 and 5) and efficiently carboxylated each protein (Table 4). These data suggest that different isoforms of the carboxylase are not required to modify these proteins *in vivo*.

These cell lines also allowed, for the first time, a comparison in activity and propeptide activation between free carboxylase (from untransfected 293 cells) and VKD protein-bound carboxylase (from transfected cells). Such a comparison has not been possible with carboxylase isolated from tissue. The carboxylase activity on a tripeptide substrate was approximately 2-fold higher in a factor IX-producing 293 cell line than in the cognate 293 cells (Table 2A,C). This increased activity appeared to be due to activation of carboxylase activity by the propeptide-containing factor IX, since the level of carboxylase protein in both cell lines was comparable (Figure 3A). The increase in carboxylase activity in factor IX 293 cells was due specifically to the propeptide since native factor IX microsomes also contained approximately 2-fold higher levels of carboxylase activity than Δpro factor IX-containing 293 microsomes (Table 2A,B) while containing the same amount of carboxylase protein (Figure 3A). *In vitro* carboxylase activity on a tripeptide substrate was stimulated by exogenously added propeptide in both 293 cells and factor IX-producing 293 cells, but the stimulation was much higher with the free carboxylase (12.5-fold vs 1.9-fold, Table 2D). The carboxylase assay was performed for 1 h, over which time the carboxylation of the peptide substrate EEL has been shown to be linear. The 12.5-fold propeptide activation observed for solubilized 293 microsomes is consistent with the fact that no endogenous VKD proteins were detected in 293 cells (Figure 2B), leaving the carboxylase freely accessible to propeptide binding. The observation that factor IX-containing microsomes only showed a 2-fold propeptide activation, however, indicates that during the 1 h assay most of the propeptide-containing factor IX remained associated with the carboxylase, possibly due to slow release of the product following carboxylation. Thus, less propeptide activation was observed in factor IX-containing microsomes than in 293 microsomes because the propeptide-containing factor IX still occupied the carboxylase propeptide binding site (Figure 7). This slow turnover of factor IX is surprising, because the turnover for the peptide substrate EEL is approximately 1/s (Table 2A). Thus, turnover of the fIX substrate *in vitro* occurred much more slowly than for the peptide substrate. The factor IX bound to carboxylase must not sterically block

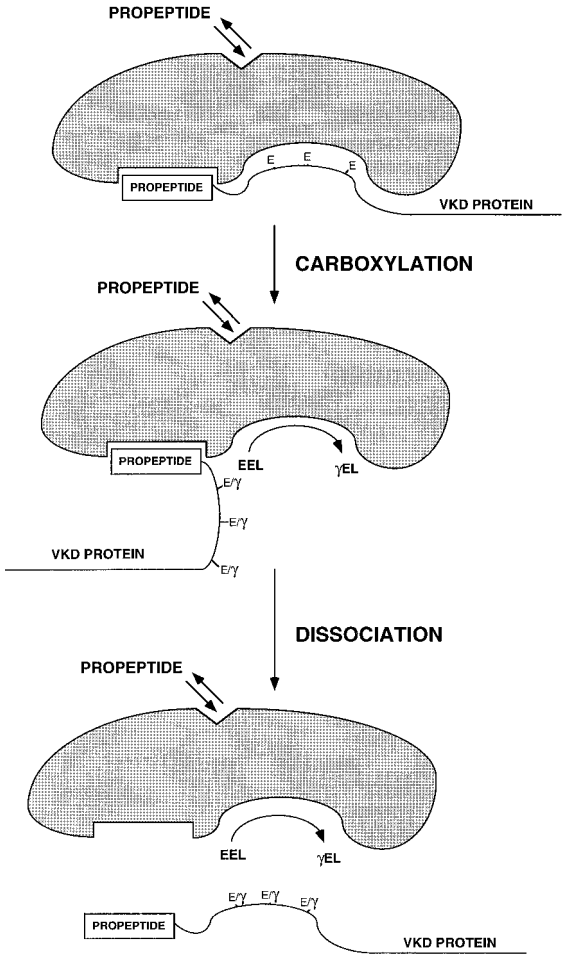

FIGURE 7: Peptide carboxylation can precede VKD protein dissociation. Two sites of carboxylase interaction with a VKD protein substrate are indicated: the propeptide binding site (indicated here by a rectangle) and the site of carboxylation (the substrate is drawn here with 3 potential Glu residues to be modified). VKD protein-bound carboxylase modifies EEL, indicating that the VKD protein does not block access of this peptide to the active site. Free carboxylase, shown at the bottom, is activated 12.5-fold by exogenously-added propeptide. However carboxylase in complex with VKD protein also exhibits propeptide activation (2-fold), and so a second potential site of propeptide activation with the carboxylase is indicated here, by a triangular cleft in the enzyme.

the active site, since EEL was efficiently carboxylated by factor IX-containing 293 microsomes. If EEL access to the active site was blocked, the free carboxylase (i.e., from untransfected 293 cells) would be predicted to have more peptide activity than bound carboxylase (from factor IX-transfected cells), but this result is the opposite of what was observed (Table 2A,C). At present, it is not known if the factor IX bound to carboxylase is fully modified during the peptide assay. We observed *in vitro* factor IX carboxylation (Figure 2B); however, quantitation was difficult with the amounts of protein studied. We are presently comparing peptide vs protein carboxylation using cell lines expressing both recombinant human carboxylase and wild type factor IX to determine whether factor IX carboxylation is completed before the onset of peptide carboxylation. Such an observation would suggest a processive mechanism for the carboxylase, i.e., in which the multiple glu residues are all modified before release by the carboxylase and subsequent modification of another substrate.

It is of interest that even the factor IX-containing 293 microsomes showed 2-fold activation by the propeptide, since

almost all of the carboxylase was shown to be in a complex with factor IX (Table 2A). A 2-fold activation was also observed with carboxylase adsorbed to immobilized anti-factor IX resin and therefore all in complex with factor IX (data not shown). We previously observed a comparable propeptide stimulation in the activity of bovine carboxylase complexed to bovine prothrombin (Berkner et al., 1992). Propeptide activation could be due to some dissociation of the VKD protein over the 1 h peptide assay period, with consequent activation of the free carboxylase by exogenous propeptide. Alternatively, the stimulation could be due to a second site of propeptide interaction, as has been proposed to account for propeptide activation observed with small peptide substrates (Knobloch & Suttie, 1987).

Isolation of the carboxylase from cell lines that produce recombinant VKD proteins also offered a novel approach for purifying and identifying this protein. Deletions or point mutations in the propeptide of protein C or factor IX, respectively, have previously been shown to abolish the carboxylation of the secreted VKD protein (Foster et al., 1987; Jorgensen et al., 1987). We used this observation to test whether there was differential binding of the carboxylase to native or Δpro factor IX. When solubilized microsomes were adsorbed onto immobilized anti-factor IX antibody, we found that the propeptide deletion in factor IX completely abolished the ability of this protein to complex with the carboxylase (Table 2). When the propeptide eluants from native factor IX or Δpro factor IX were subsequently analyzed by gel electrophoresis, an apparent single 98 kDa form was observed, only from native factor IX-derived microsomes (Figure 5A).

The 98 kDa band that specifically bound to native factor IX comprised two different proteins as determined by microsequence analysis and Westerns (Table 3, Figure 6). One protein corresponded to the human homologue of the 95 kDa bovine liver carboxylase isolated by Wu et al. (1991b), and the other corresponded to the homologue of a 98 kDa bovine liver protein that we isolated (Berkner et al., 1992) and have called the γ-carboxylase-associated protein or GCAP (Berkner et al., unpublished data). cDNAs encoding these two proteins predicted substantially different sequences, with no apparent homology (Berkner et al., unpublished data; Wu et al., 1991a). These two proteins were isolated only from 293 cells expressing native factor IX, demonstrating their specificity for a molecule containing both the propeptide and the gla domain (Figure 6). The stoichiometry of the two proteins is not known, as neither the Western nor microsequence analysis is quantitative. In addition, while the carboxylase isolation could be monitored throughout the purification, there was no assay for GCAP, and it is not known how labile the association of the two proteins is.

GCAP most likely associates directly with the carboxylase. First, GCAP is only isolated in the purification from cells expressing native factor IX, and not the Δpro factor IX (Figure 6A). Second, affinity purified anti-peptide antisera raised to this protein immunocaptured carboxylase activity both from crude microsomes and from purified carboxylase that did not contain detectable levels of VKD proteins (Berkner et al., unpublished data). We have expressed cDNAs encoding GCAP or the carboxylase in insect cells and in mammalian cells either that do not contain VKD proteins or that coexpress undercarboxylated recombinant factor IX (Berkner et al., unpublished data). Only the carboxylase cDNA encodes catalytic activity on a small peptide substrate in infected insect cells. Both cDNAs effect increased carboxylase peptide activity in transfected mammalian cells; however, the carboxylase cDNA encodes substantially more peptide activity. At present, the function of GCAP is not known. This protein may play a specific role in the carboxylation of the VKD proteins. Alternately, GCAP may function in the secretion of the carboxylated VKD proteins or may specifically fulfill some carboxylase requirement, e.g., in its cellular localization or stability.

The identification of potential factors that may interact with the carboxylase intracellularly is complicated by the unusual requirements of the carboxylase for isolation: both phospholipid and detergent are necessary to retain activity, so that the carboxylase is actually purified in a micelle. Isolating proteins that copurify with the carboxylase thus requires a compromise between specificity and recovery. Approaches such as the use of detergents (Wu et al., 1991b) or increased salt concentrations (Figure 5) in column washes facilitate an increase in the specific activity of the isolated carboxylase, but may also strip off proteins specifically bound to the carboxylase. With native factor IX and with Δpro factor IX, additional proteins were observed when the factor IX resin was washed under less stringent conditions (Figure 5C,D). The functional significance of these additional proteins, however, is unclear. For example, two functionally irrelevant proteins copurified in abundance with Δpro factor IX: a nuclear protein involved in transcription and a putative splicing factor. These proteins did not copurify with native factor IX. Thus their copurification with Δpro factor IX may be related to the abnormal structure of this protein, or their presence may be an artifact due to isolating proteins in micelles. Therefore, the isolation of other carboxylase associated proteins that play a role in VKD protein secretion may be difficult, and their identification may require a different approach.

The isolation of the carboxylase from human tissue culture cell lines provides a useful framework for studies on the intracellular mechanisms of γ-carboxylation. Analysis of the binding and modification of intracellular recombinant factor IX by the carboxylase should be useful in studying the limiting step(s) in the secretion of carboxylated VKD proteins. In addition, the isolation described here should be adaptable for purifying recombinant carboxylases. Purification from a factor IX−carboxylase complex allowed nearly quantitative recovery of the carboxylase. Purification from cell lines coexpressing recombinant carboxylase and recombinant VKD protein should therefore overcome the barrier previously observed in purifying recombinant carboxylase (Kuliopulos et al., 1994). We have optimized conditions so that a near-homogeneous preparation of protein can be obtained using a single affinity purification. This procedure should be useful for purifying variants of the carboxylase for biochemical analysis and for characterizing intracellular interactions of the carboxylase with other proteins.

ACKNOWLEDGMENTS

The authors would like to thank Kathy Walker for synthesizing the peptides used in these experiments, Don Foster for providing the two protein C cell lines used here,

and Beth McNally for her assistance in some of the experiments. We would like to thank Kurt Runge for valuable comments in the preparation of the manuscript.

REFERENCES

Aebersold, R. H., Leavitt, J., Saavedra, R. A., Hood, L. E., & Kent, S. B. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6970−6974.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990) *J. Mol. Biol. 215*, 403−410.

Berkner, K. L. (1993) *Methods Enzymol. 222*, 450−477.

Berkner, K., Busby, S., Davie, E., Hart, C., Insley, M., Kisiel, W., Kumar, A., Murray, M., O'Hara, P., Woodbury, R., & Hagen, F. (1986) *Cold Spring Harbor Symp. Quant. Biol. 51*, 531−541.

Berkner, K. L., Boel, E., & Prunkard, D. (1988) in *Viral Vectors*, pp 56−61, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Berkner, K. L., Harbeck, M., Lingenfelter, S., Bailey, C., Sanders-Hinck, C. M. & Suttie, J. W. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6242−6246.

DeMetz, M., Vermeer, C., Soute, B. A. M., Van Scharrenburg, G. J. M., Slotboom, A. J., & Hemker, H. C. (1981) *FEBS Lett. 123*, 215−218.

Dong, B., Horowitz, D. S., Kobayashi, R., & Krainer, A. R. (1993) *Nucleic Acids Res 21*, 4085−4092.

Foster, D. C., Rudinski, M. S., Schach, B. G., Berkner, K. L., Kumar, A. A., Hagen, F. S., Sprecher, C. A., Insley, M. Y., & Davie, E. W. (1987) *Biochemistry 26*, 7003−7011.

Foster, D. C., Sprecher, C. A., Holly, R. D., Gambee, J. E., Walker, K. M., & Kumar, A. A. (1990) *Biochemistry 29*, 347−354.

Fraser, J. D., & Price, P. A. (1988) *J. Biol. Chem. 263*, 11033−11036.

Friedman, P. A., & Przysiecki, C. T. (1987) *Int. J. Biol. Chem. 19*, 1−7.

Furie, B., & Furie, B. C. (1988) *Cell 53*, 505−518.

Furie, B., & Furie, B. C. (1990) *Blood 75*, 1753−1762.

Gower, H. J., Moore, S. E., Dickson, G., Elsom, V. L., Nayak, R., & Walsh, F. S. (1989) *Development 105*, 723−731.

Grinnell, B. W., Berg, D. T., Walls, J., & Yan, S. B. (1987) *Biotechnology 5*, 1189−1192.

Harlow, E., & Lane, D. (1988) *Antibodies: A laboratory manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Hubbard, B. R., Ulrich, M. M. W., Jacobs, M., Vermeer, C., Walsh, C., Furie, B., & Furie, B. C. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6893−6897.

Jorgensen, M. J., Cantor, A. B., Furie, B. C., Brown, C. L., Shoemaker, C. B., & Furie, B. (1987) *Cell 48*, 185−191.

Knobloch, J. E., & Suttie, J. W. (1987) *J. Biol. Chem. 262*, 15334−15337.

Kuliopulos, A., Nelson, N. P., Yamada, M., Walsh, C. T., Furie, B., Furie, B. C., & Roth, D. A. (1994) *J. Biol. Chem. 269*, 21364−21370.

Manfioletti, G., Brancolini, C., Avanzi, G., & Schneider, C. (1993) *Mol. Cell Biol. 13*, 4976−4985.

Munro, S., & Pelham, H. R. B. (1986) *Cell 46*, 291−300.

Patton, J. G., Porro, E. B., Galceran, J., Tempst, P., & Nadal-Ginard, B. (1993) *Genes Dev. 7*, 393−406.

Price, P. A. (1988) *Annu. Rev. Nutr. 8*, 565−583.

Rehemtulla, A., Roth, D. A., Wasley, L. C., Kuliopulos, A., Walsh, C. T., Furie, B., Furie, B. C., & Kaufman, R. J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 4611−4615.

Roth, D. A., Rehemtulla, A., Kaufman, R. J., Walsh, C. T., Furie, B., & Furie, B. C. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 8372−8376.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Suttie, J. W. (1985) *Annu. Rev. Biochem. 54*, 459−77.

Varnum, B. C., Young, C., Elliott, G., Garcia, A., Bartely, T. D., Fridell, Y.-W., Hunt, R. W., Trail, G., Clogston, C., Toso, R. U., Yanagihara, D., Bennett, L., Sylber, M., Merewether, L. A., Tseng, A., Escobar, E., Liu, E. T., & Yamane, H. K. (1995) *Nature 373*, 623−626.

Vermeer, C. (1990) *Biochem. J. 266*, 625−636.

Wu, S. M., Cheung, W. F., Frazier, D., & Stafford, D. W. (1991a) *Science 254*, 1634−1636.

Wu, S. M., Morris, D. P., & Stafford, D. W. (1991b) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2236−2240.

Yang, Y. S., Hanke, J. H., Carayannopoulos, L., Craft, C. M., Capra, J. D., & Tucker, P. W. (1993) *Mol. Cell. Biol. 13*, 5593−5603.

BI9523318