Inhibitors of 2-Ketoglutaryl-dependent Dioxygenases Block Aspartyl β-Hydroxylation of Recombinant Human Factor IX in Several Mammalian Expression Systems*

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While a role has been ascribed to the γ-carboxyglutamate (Gla) residues in vitamin K-dependent coagulation proteins and the enzyme catalyzing this post-translational modification has been identified and partially characterized, both the functional significance of a second posttranslationally synthesized amino acid found in these proteins, β-hydroxyaspartate (Hya), and the aspartyl β-hydroxylating enzyme remain to be determined. We now report on inhibitors of 2-ketoglutaryl-dependent dioxygenases, such as dipyrrolidyl-ephedranol, and pyridine 2,4-dicarboxylate, block hydroxylation of Asp64 in recombinant factor IX molecules produced in three different mammalian expression systems. This hydroxylation was not inhibited by the specific copper chelators 2,9-dimethylphenanthroline or D-penicillamine. The Gla levels in these proteins were unaffected by these compounds and demonstrate that carboxylation proceeds independently of hydroxylation. Using these Hya-deficient recombinant factor IX molecules we demonstrate that this residue does not play a significant role in factor IX binding to endothelial cells under equilibrium conditions. From additional binding studies we have concluded that the Gla domain of factor IX is a major cell binding domain of factor IX. Furthermore, in contrast to studies demonstrating a marked loss of one-stage clotting activity in recombinant factors IX following site-directed mutations of Asp46 to neutral or basic residues (Rees, D. J. G., Jones, I. M., Handford, P. A., Walter, S. J., Esnouf, M. P., Smith, K. J., and Brownlee, G. J. (1988) EMBO J. 7, 2053–2061), we have not found a decrease of one-stage clotting activity with Hya-deficient factor IX. Hya-deficient proteins produced in this manner may prove to be more appropriate to elucidate the function of Hya than those produced by site-directed mutagenesis.

Factor IX is a plasma glycoprotein belonging to the family of vitamin K-dependent coagulation proteins. It, as well as a number of other vitamin K-dependent proteins, including factor VII, factor X, protein C, and protein S, contain two known posttranslationally modified amino acids found in discrete regions of the molecule (see Ref. 1 for a review; 2, 3). The amino terminus of factor IX is rich in γ-carboxyglutamatic acid (Gla) residues; these are formed by the action of the vitamin K-dependent γ-glutamyl carboxylase on specific glutamyl residues and appear to confer upon this domain its capacity to bind calcium and interact with phospholipids (4). The Gla domain is followed by two epidermal growth factor (EGF)-like domains, the first of which contains one β-hydroxyaspartic acid (Hya) residue at position 64. The functional role(s) of the EGF homology regions and the significance of the Hya residue in particular are not well understood. It has been suggested that the EGF homology regions play a role in protein-protein or protein-cell interactions (6, 7). Site-directed mutagenesis of residue 64 to Lys, Val, or Gly results in factor IX with markedly decreased clotting activity (8). Such studies, while strongly suggesting that this EGF-like domain is essential for factor IX function, do not directly address the functional significance of the posttranslational hydroxylation. This might better be accomplished by comparing the functions of factor IX molecules having either Asp or Hya at residue 64. To this end, we have observed that several compounds which inhibit 2-ketoglutaryl dioxygenases also block β-hydroxylation of Asp46 of recombinant human factor IX. These Hya-deficient molecules provide a starting point for the elucidation of the functional significance of the hydroxylation.

**EXPERIMENTAL PROCEDURES**

Cell Culture—CHO cells were cultured as described previously (9). BHK cells and human kidney cell line 293 (10) were routinely grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1% penicillin/streptomycin, 1 mg/ml G418, and 5 µg/ml vitamin K1 (11). Bovine aortic endothelial cells were cultured as described previously (12).

Production of Recombinant Factor IX—Confluent CHO, BHK, or 293 cells were incubated with harvest media for 24 h, at which time the media were collected, adjusted to 1 mM benzamidine, centrifuged to remove cellular debris, and either subjected to immediate purification or stored at −20°C for future processing. CHO cell-derived factor IX was harvested in medium containing 1 mg/ml bovine serum albumin, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium, 100 µg/ml putrescine, 10 mM hydrocortisone, and 5 µg/ml vitamin K1.

1 Protein S is unique among these proteins in possessing a third such modification, β-hydroxyasparaginase, as well as four epidermal growth factor-like domains. The first epidermal growth factor-like domain contains β-hydroxyaspartic acid while the next three contain β-hydroxyasparaginase (6).

2 The abbreviations used are Gla, γ-carboxyglutamatic acid; Hya, β-hydroxyaspartic acid; CHO, Chinese hamster ovary; BHK, baby hamster kidney; EGF, epidermal growth factor.
BHHC and 293-derived factor IX were harvested in medium containing 1% fetal bovine serum and 5 μg/ml vitamin K₁.

Treatment of Cells with Inhibitors—Dipyridyl, o-phenanthroline, and 2,9-dimethylphenanthrolines were dissolved in 70% ethanol, n-Penicillamine, pyridine 2,4-dicarboxylate, and pyridine 3,4-dicarboxylate were solubilized in deionized water. When cells were to be incubated with inhibitors, they were routinely exposed to the medium containing the inhibitor for 24 h prior to protein harvesting. In some studies, cells were incubated for 6–8 h with inhibitor-containing medium, and then fresh medium containing inhibitor was added, followed by a harvest period of 18–24 h.

Purification of Factor IX—Unless otherwise indicated, factor IX in culture supernatants was first adsorbed to barium sulfate and then eluted in 0.17 M sodium citrate. The eluted factor IX was isolated by immunoaffinity chromatography as described by Ahmad et al. (13). Proteins were dialyzed into ammonium bicarbonate for characterization.

Protein Characterization—Purity of proteins was evaluated by sodium dodecyl sulfate-gel electrophoresis on 8–25% polyacrylamide gels using PhastSystem (14). Proteins were identified by silver staining. NH₄-terminal sequence was obtained by automated Edman degradation. High performance liquid chromatography quantitation of Gla and Hya content was made on 10–25 Kg of purified protein after either base or acid hydrolysis, respectively (6).

Binding Assay—Confluent endothelial cells were incubated with 4 nM human factor IX for 2 h at 4 °C as described by Stern et al. (15). When present, increasing concentrations of unlabeled competitor were added immediately before the radioligand. Non-specific binding was designated as the amount bound in the presence of a 100-fold excess of unlabeled factor IX.

Preparation of Factor IXa, Gla-domainless Factor IXa, Factor IX, Gla Peptide, and Des-carboxy Factor IX—Factor IXa was prepared from plasma-isolated human factor IX by activation with factor Xa (16). Gla-domainless factor IXa and the Gla peptide were prepared by limited chymotryptic digestion as described previously (17). Recombinant des-carboxy factor IX was isolated from CHO cells incubated with sodium Warfarin (1 μg/ml) for 24 h.

RESULTS AND DISCUSSION

Recombinant human factors IX, expressed in three different cell lines (CHO, BHHC, 293), were purified to homogeneity. The recombinant factors migrated as single broad bands each with an apparent mass of 66 kDa on sodium dodecyl sulfate gels similar to factor IX isolated from human plasma. Sequence analysis revealed that CHO cell-derived factor IX was incompletely processed at the NH₂ terminus with approximately 50% of the secreted protein still containing the 18-residue prepeptide sequence. The factors IX secreted by both kidney cell lines were greater than 85% fully processed, as described previously (18). The Gla and Hya content of these proteins were compared to those of plasma factor IX. Factor IX isolated from the CHO cells by immunoaffinity chromatography without prior barium adsorption had 2–3 mol of Gla/mol protein. If a barium adsorption step was included in the purification, this increased to nearly 5 mol of Gla/mol protein (Table I); thus, this factor IX still possessed only 50% of the Gla content measured by us for plasma factor IX, 10–11 mol of Gla/mol protein. By contrast, the factor IX obtained from either the BHHC or 293 cell lines after barium adsorption and immunoaffinity chromatography was more fully carboxylated (9 mol of Gla/mol protein) (Table I). The CHO cells produce 10–20 times more factor IX than do the BHHC or 293 cell lines;

thus, it is possible that the undercarboxylation seen with the CHO cells occurs because the γ-glutamyl-carboxylating system becomes saturated. Analyses of acid hydrolysates of each of the three recombinant factors X suggest that Asp is only partially hydroxylated (0.34–0.55 mol of Hya/mol protein) (Table I). These values are somewhat elevated compared to our determinations for plasma factor IX (0.24 ± 0.02 mol of Hya/mol protein) but are within the range reported by others for plasma and recombinant human factor IX (19, 20).

The cofactor requirements for Asp hydroxylase are undefined. We examined a number of known inhibitors of 2-ketogluurate-dependent dioxygenases and dopamine β-hydroxylase for their abilities to inhibit hydroxylation of factor IX expressed in CHO cells. Table II summarizes these results. Both dipyridyl and o-phenanthroline, agents that chelate either Fe²⁺ or Cu²⁺, inhibited Asp β-hydroxylase of CHO cell-derived factor IX. With either chelator, factor IX isolated from conditioned media without a preincubation period showed about an 80% reduction of Hya levels in a 24-h harvest. If, after an initial 8-h preincubation, fresh medium containing inhibitor was added, the cells were washed and fresh medium containing the inhibitor were made only on the factor IX secreted over the ensuing 18–24 h, Hya levels were reduced by more than 95%. Similar levels of inhibition of hydroxylation were seen in factors IX from dipyridyl-treated BHHC or 293 cells (data not shown). The further decrease observed after preincubation probably reflects, at least in part, the secretion during the preincubation period of already hydroxylated factor IX molecules still trafficking the cell. Since neither p-penicillamine (500 μM) nor 2,9-dimethylphenanthrolines (100 μM), selective copper chelators and potent inhibitors of the copper-dependent dopamine β-hydroxylase (21, 22), block hydroxylation of Asp (data not shown), Asp β-hydroxylase may be more like the Fe²⁺-dependent 2-ketogluurate dioxygenases. To further evaluate this possibility, we studied the effects of the putative 2-ketogluurate cosubstrate inhibitor pyridine 2,4-dicarboxylate (23, 24).

When this compound was present at an initial concentration of 5.5 mM, a level which effectively inhibits Fe²⁺-dependent 2-ketogluurate dioxygenases and dopamine β-hydroxylase, 50% of the Gla content was measured by us for plasma factor IX, 10–11 mol of Gla/mol protein. By contrast, the factor IX obtained from either the BHHC or 293 cell lines after barium adsorption and immunoaffinity chromatography was more fully carboxylated (9 mol of Gla/mol protein) (Table I). The CHO cells produce 10–20 times more factor IX than do the BHHC or 293 cell lines;

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### Table I

| Gla and Hya content of recombinant human factor IX isolated from several mammalian expression systems | Gla mol/mol protein | Hya mol/mol protein |
|-------------------------------------------------|---------------------|---------------------|
| Plasma factor IX                               | 10.62 ± 0.27        | 0.24 ± 0.02         |
| CHO factor IX                                  | 4.82                | 0.40                |
| BHHC factor IX                                 | 9.46                | 0.34                |
| 293 factor IX                                  | 9.28                | 0.55                |

### Table II

| Treatment               | Concentration | Hya mol/mol factor IX |
|-------------------------|---------------|-----------------------|
| Control                 |               | 0.50 ± 0.08 (13)      |
| 2,2'-Dipyridyl          | 100 μM        | 0.08 ± 0.02 (4)*      |
| o-Phenanthroline        | 100 μM        | 0.07 ± 0.02 (2)*      |
| Pyridine 2,4-dicarboxylate | 5.5 mM       | 0.22 ± 0.04 (4)*      |
| Pyridine 3,4-dicarboxylate | 5.5 mM       | 0.49 (1)              |
| 2,2'-Dipyridyl          | 100 μM        | 0.02 ± 0.01 (4)*      |
| Pyridine 2,4-dicarboxylate | 5.5 mM       | 0.09 (1)              |

* p < 0.01.
* p < 0.05.
position 2 (24). Pyridine 3,4-dicarboxylate, which has chelating and prolyl hydroxylase inhibitory activities that are 1000-fold less than the 2,4-isomer (24), was not an inhibitor of Asp β-hydroxylation (Table II).

Warfarin effectively blocks vitamin K-dependent γ-glu-tamyl carboxylation in vivo by inhibiting vitamin K reductases. Since in cell culture hydroxylation proceeds when Warfarin is present, hydroxylation is independent of carboxylation (20). We have confirmed this observation in our studies (data not shown). Inhibition of Asp β-hydroxylation by dipyrindyl treatment did not significantly affect the Gla levels of these proteins (control BHK: Gla, 9.4 mol, Hya, 0.34 mol; dipyrindyl-treated BHK: Gla, 9.2 mol, Hya, 0.02 mol). Thus, the converse is true: carboxylation is independent of hydroxylation.

The physiological roles of neither the EGF-like domain nor of Hya are fully understood. A mutant recombinant human protein C in which Hya1 was replaced with Glu has been shown to lack a Ca2+-dependent epitope and to have only 10% of normal biological activity (25). We have noted that the 4 residues NH2-terminal to the first cysteine of the EGF-like domains in coagulation factors VII, IX, X, and protein C are identical (6). The region is acidic, having the sequence Asp47-Gly48-Asp49-Gln50. With the cysteine pairing of EGF (26), these residues may be brought into close proximity to the (potentially) hydroxylated Asp46, Ca2+ binding and biological activity of the EGF-like domain could be mediated by the interactions of Asp47, Asp49, and Asp64, perhaps with the involvement of the hydroxyl group. Indeed, mutant recombinant human factor IX (8) in which Asp64 was replaced with either a neutral or basic residue (Gly, Val, Lys) retained <2% of the one-stage clotting activity of normal factor IX. Replacement of Asp47 with Lys or Gly gave molecules with 1 or 5%, respectively, of normal clotting activity, while replacement of Asp49 with Glu resulted in a molecule with 8% of normal activity. In preliminary experiments comparing the one-stage clotting activities of essentially unhydroxylated factors IX from CHO, BHK, or 293 cells with their respective hydroxylated counterparts, we have not detected any significant differences (data not shown). Taken together, these data are compatible with the importance of the acidic nature of residues 47, 49, and 64; furthermore, they suggest that with respect to clotting activity the special functional significance (if any) of the cohort of plasma factor IX which is β-hydroxylated is more subtle than would be expected from one-stage clotting assays.

There is evidence suggesting that hydroxylated EGF-like domains mediate the interaction between complement proteins C1r and C1s (27) and possibly the interaction between proteins C and S (25). Factor IX has been shown to bind to endothelial cell receptors in a calcium-dependent and -specific manner (15). In preliminary studies, it was reported both that Gla-domainless factor IX bound to cultured endothelial cells (28), and that the decapetide sequence in factor IX, NH2-terminal to the first cysteine of the EGF-like domain and including the acidic tetrapeptide sequence, inhibited factor IX binding to endothelial cells, albeit with a Ki of 200 μM (29). Thus, it was appropriate to evaluate the role of the EGF-like domain, and, more specifically, the role of Hya in the binding of factor IX to endothelial cells. We determined that either recombinant factor IX isolated from control (+Hya) or dipryridyl-treated (−Hya) BHK cells competed as well as plasma factor IX (IC50 = 8 nM) against 125I-plasma factor IX for specific binding to cultured bovine aortic endothelial cells when binding was measured under equilibrium conditions (Fig. 1). Similar results were obtained with proteins isolated from the 293 cell line while CHO cell-derived factor IX competed but with an IC50 of 30 nM (data not shown). This higher value may represent the lower Gla content of the CHO cell factor IX as well as the incomplete processing. Thus, Hya does not appear essential for factor IX binding under these conditions. We have not yet evaluated the possibility that the presence of Hya affects association or dissociation rate constants. We next examined the relative importance of the Gla and EGF-like domains for binding. Intact proteins as well as fragments of plasma factor IX were studied. Factor IX and factor IXa competed with radiolabeled plasma factor IX with IC50 values of 8 and 3 nM, respectively (Table III). This severalfold increase in binding affinity of factor IXa has been reported (30). Factor IXa-derived Gla peptide was also an effective competitor with an IC50 of 30 nM. Removal of the Gla domain by chymotryptic digestion (Gla-domainless factor IXa) or production of des-carboxy factor IX (isolated from Warfarin-treated CHO cells) resulted in proteins that could not compete for factor IX binding to cultured endothelial cells (Table III). From these results it would appear that the Gla domain provides much of the binding energy for this interaction. Since inhibitors of 2-ketogluatrate dioxygenases block Asp β-hydroxylation, the enzyme catalyzing this activity may resemble prolyl and lysyl hydroxylases. Furthermore, if our hypothesis (6) that not only the acidic nature of residues 47, 49, and 64 may be important, then it is possible that the acidic motif in the EGF-like domain could mediate a specific interaction to endothelial cells.

![Fig. 1. Recombinant factor IX (±Hya) competes with plasma factor IX for binding to endothelial cells. Human 125I-factor IX (4 nM) was incubated with confluent endothelial cells in the presence of 0.25–400 nM unlabeled competitor for 2 h at 4 °C. Maximal binding (100%), which was defined as total binding minus nonspecific binding obtained in the presence of a 100-fold molar excess of factor IX, corresponded to 93 ± 7 fmol/35-mm dish. Nonspecific binding averaged 20–30%; •, human plasma factor IX; ○, factor IX isolated from control BHK cells; O, factor IX isolated from dipyrindyl-treated BHK cells (0.02 ± 0.01 mol of Hya/mol factor IX).](image)

| Unlabeled competitor | IC50 (nM) |
|----------------------|----------|
| Factor IX            | 8        |
| Factor IXa           | 3        |
| Factor IXa Gla peptide | >400    |
| Gla-domainless factor IXa | >400    |

| Table III. Inhibition of plasma factor IX binding to cultured endothelial cells by derivatives of factor IX |

Binding assays were performed as described in Fig. 1.
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49, and 64 but also the length of their acidic side chains are vital to the function of a Hya-containing EGF-like domain, then the use of these inhibitors and not site-directed mutagenesis in recombinant cell expression systems may be more appropriate for elucidating the function(s) of Hya (or β-hydroxyasparagine) in proteins containing these modifications.

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