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Dexamethasone-Induced Phospholipase A_2-Inhibitory Proteins (PLIP) Influenced by the *H-2* Histocompatibility Region (41980)

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Abstract. Recent work has indicated that the *H-2* histocompatibility complex on chromosome 17 influences the degree of glucocorticoid-induced teratogenicity and anti-inflammatory response. Since both of these hormonal actions appear to be mediated by the induction of phospholipase A_2-inhibitory proteins (PLIP), the influence of the *H-2* complex on the induction of PLIP by glucocorticoids in thymocytes and embryonic palates has been investigated. Analysis of dexamethasone-induced PLIP by Sephadex G-100 revealed four peaks of mol wt 55,000, 40,000, 28,000 and 15,000 in mouse thymocytes and from one to three of these PLIPs in mouse embryonic palates. The 55,000 mol wt PLIP comprised 50–60% of the total activity. The total amount of dexamethasone-induced PLIP is significantly higher in B10.A ($H\text{-}2^a$) thymocytes than that in thymocytes of their congenic resistant partners, B10 ($H\text{-}2^b$). The induced level of PLIP in the embryonic palates treated with dexamethasone is also significantly higher in the $H\text{-}2^a$ congenic strains with either the A or B background (AWy or B10.A) than that in their resistant partners (A.BY or B10). Thus, both susceptibility to glucocorticoid-induced cleft palate and the production of PLIP by this hormone are influenced by the *H-2* complex.

Susceptibility of mice to cortisone-induced cleft palate is influenced by genes linked to the *H-2* histocompatibility locus (1–4). Evidence (5–8) from our laboratory has suggested that the teratogenic action of glucocorticoids in producing cleft palate in rodents utilizes the same biochemical mechanism as that by which glucocorticoids produce their anti-inflammatory effects. The anti-inflammatory pathway of glucocorticoids involves a receptor-mediated production of phospholipase A_2-inhibitory proteins which inhibit the release of arachidonic acid with a subsequent diminished production of prostaglandins and thromboxanes by cyclooxygenase and of leukotrienes by lipoxygenase (9–12). Similar mechanisms appear to be involved in the induction of cleft palate, as exogenous arachidonic acid injected into pregnant rats (6) or mice (13) at the same time as glucocorticoids markedly reduces the teratogenic potency of the steroids and indomethacin, an inhibitor of cyclooxygenase, blocks the corrective action of arachidonic acid (6, 13, 14). Moreover, as would be expected, in addition to susceptibility we have shown that the *H-2* complex influences glucocorticoid receptor content and the degree of inhibition of arachidonic acid release and prostaglandin biosynthesis in thymocytes and embryonic palates (15). Furthermore, we have demonstrated that PLIPs induced by glucocorticoids in various tissues, including the embryonic palate, are capable of mediating the teratogenic effects of glucocorticoids *in vitro*, i.e., inhibition of programmed cell death in the medial edge epithelium of the embryonic palate (16). In this report we have compared the production of PLIP induced by dexamethasone in thymocytes and embryonic palates of susceptible ($H\text{-}2^a$) and resistant ($H\text{-}2^b$) congenic mouse strains which vary in the *H-2* locus but otherwise are virtually identical in genetic background. We show here that glucocorticoid-induced PLIP is significantly higher in thymocytes of the B10.A ($H\text{-}2^a$) strain than in those of their congenic resistant partner, B10 ($H\text{-}2^b$). We also show that the production of PLIP in the embryonic palates of $H\text{-}2^a$ congenic mouse strains with either the A or B10 background (AWy and B10.A) is significantly higher than in those of their resistant $H\text{-}2^b$ partners (A.BY and B10).

Materials and Methods. *Animals.* Inbred and congenic mouse strains (AWy, A.BY, B10.A, and B10) were obtained from the Jackson Laboratory, Bar Harbor, Maine, and were fed Purina mouse chow. Female mice

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were weighed and mated overnight with the appropriate males. The females having plugs the next morning were considered pregnant as Day 0 of gestation. For studies using embryonic palates, embryos were used on Day 14 of gestation. The embryonic tissue was obtained by cutting the head horizontally below the level of the eyes and included the palate and upper and lower jaws. In other studies, thymuses were used from adult male mice.

Preparation of PLIP. PLIP was produced from mouse thymus or embryonic palates using a modification of the procedure of Hirata *et al.* (10). Thymocytes from eight mice or 20 embryonic palates were incubated with or without 50 μM dexamethasone in 5 ml of RPMI 1640 containing 0.1% BSA and 50 μl of streptomycin–penicillin solution (100 units of penicillin and 100 mg of streptomycin–penicillin solution (100 units of penicillin and 100 mg of streptomycin/ml) at 37°C for 16–18 hr. After incubation, the medium was removed following centrifugation at 1000*g* and discarded. The sediment was treated with 0.5 ml of 2% Nonidet solution for 30 min and the supernatant was separated and used for the preparation of PLIP using Sephadex G-100 column chromatography (2 × 30-cm column). PLIP was eluted by 50 m*M* Tris–HCl buffer and 2-ml fractions were collected. The PLIP activity was determined in all fractions using the procedure described below. The concentrations of proteins in all fractions were determined from their absorbance at 280 nm. Typical examples of elution patterns of proteins in thymus and embryonic palates are shown in Fig. 3.

Assay of PLIP. PLIP was assayed by its ability to inhibit snake (*Naja naja naja*) venom phospholipase A_2 (PLA_2) using the procedure described elsewhere (10) with some modification. The activity of phospholipase A_2 was measured *in vitro* with and without PLIP. The reaction mixture contained, in a total volume of 1 ml, 50 m*M* Tris buffer, pH 8.0, 0.1 unit of snake venom phospholipase A_2, 20,000 dpm of L-α-phosphatidylcholine and 1-stearoyl-2-[1-^{14}C]arachidonyl (59.3 mCi/mmole) and incubated at 37°C for 15 min. The reaction was stopped by adding 6 ml of hexane in the incubation tubes and each tube was vortexed for 10 sec. [^{14}C]Arachidonic acid released by phospholipase A_2 was extracted into the hexane layer. Three milliliters of the hexane layer was counted and taken as a measure of the activity of phospholipase A_2. One unit of *N. naja naja* snake venom PLA_2 hydrolyzes 1 μmole of L-α-phosphatidylcholine to L-α-lysophosphatidylcholine and a fatty acid ([^{14}C]arachidonic acid) at pH 8.0 at 25°C. PLIP activity was determined from the percentage inhibition of snake venom PLA_2. One unit of PLIP is defined as the amount of PLIP that inhibits one unit of *N. naja naja* snake venom PLA_2.

Statistics. Statistical significance was calculated by Student's *t* analysis.

Results. *Thymocytes.* Analysis of dexamethasone-induced PLIP by Sephadex G-100 column chromatography demonstrated four peaks of mol wt, 55,000, 40,000, 28,000, and 15,000 (Fig. 1). The amounts of the various-sized PLIP produced by the mouse thymocytes in the presence and absence of dexamethasone are shown in Table I. The results show that control mouse thymocytes have an appreciable amount of PLIP in the absence of dexamethasone which is significantly higher in B10.A thymocytes than in B10. PLIP is significantly induced by the treatment with dexamethasone. The 55,000 mol wt PLIP fraction comprised 50–60% of the total PLIP activity indicating that this is the major PLIP fraction and this component, as well as the 15,000 mol wt component, was significantly induced by dexamethasone ($P < 0.01$). In some experiments we found very little or no production of the small molecular weight components. The dexamethasone-induced levels of total PLIP, the 55,000, 28,000, and 15,000 mol wt fractions, were significantly higher in B10.A than in B10, thus showing the influence of the *H-2* locus in the induction of PLIP by dexamethasone. The protein concentration in the various fractions of column chromatography as shown in Fig. 3 remained unchanged following treatment with dexamethasone (data not shown).

Embryonic palates. The induction of PLIP by treatment with dexamethasone in the embryonic palates of the various congenic strains is shown in Table II, Table III, and Fig. 2. The levels of both control and dexa-

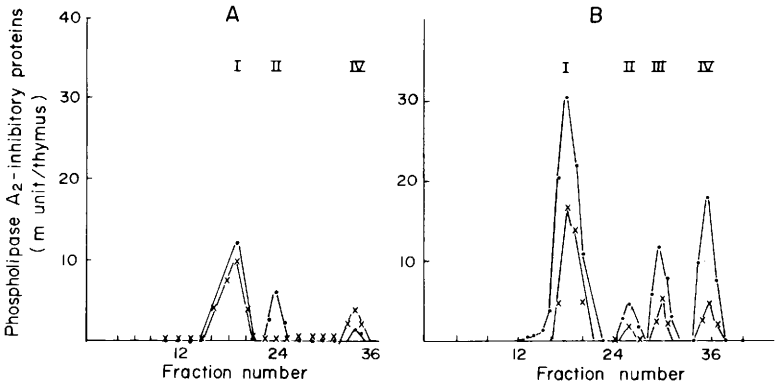

FIG. 1. Analysis of PLIP prepared from mouse thymocytes by Sephadex G-100 in the presence (●) and in the absence of dexamethasone (×) in B10 (A) and B10.A (B) is shown. I, II, III, and IV peaks designate 55,000, 40,000, 28,000, and 15,000 PLIPs. The definition of the unit of PLIP is given in Table I.

methasone-induced PLIP are much lower in the embryonic palate than in the adult thymus (Tables I and II). Activity of PLIP was found exclusively in the 55,000 mol wt fraction of B10.A and B10 palates (Table II), whereas the activity of PLIP in the AWy and A.BY palates occurs in the 55,000, 28,000, and 15,000 mol wt fractions (Table III). The total activities of PLIP and the 55,000 mol wt fraction induced by dexamethasone were significantly higher in B10.A and AWy compared to their corresponding *H-2* partners, B10 and A.BY, respectively.

Discussion. The results of the present study demonstrate that dexamethasone induces the synthesis of proteins exhibiting the antiphospholipase A_2 properties of glucocorticoids in the thymus and embryonic palates and that the *H-2* gene complex influences the degree of this induction. Previously, we have demonstrated that *H-2*-linked susceptibility to glucocorticoid-induced cleft palate is correlated with the *H-2*-linked difference in the glucocorticoid-receptor content and the degree of glucocorticoid-induced anti-inflammatory response (15). Thus, the present results support the concept that the *H-2* complex influences the amount of glucocorticoid-receptor protein as the rate-limiting step of the biochemical pathway of cleft palate induction by glucocorticoid which subsequently influences the production of

TABLE I. INDUCTION OF PHOSPHOLIPASE A_2-INHIBITORY PROTEINS BY DEXAMETHASONE IN B10.A AND B10 THYMOCYTES

	Dexamethasone (munit/thymus)			
	B10.A		B10	
PLIP mol wt	Without	With	Without	With
Total	97.0 ± 45.0	186.0 ± 32.0[a] (92)	13.0 ± 13.0[b]	17.0 ± 11.5[b] (31)[a]
55,000	58.0 ± 37.0	98.0 ± 25.0[a] (69)	10.0 ± 13.0[b]	12.0 ± 9.0 (20)
40,000	10.7 ± 10.1	15.8 ± 8.5 (48)	0.0 ± 0.0[b]	1.7 ± 3.3
28,000	16.0 ± 10.7	32.7 ± 18.6 (104)	0.0 ± 0.0[b]	0.5 ± 1.0[b]
15,000	11.3 ± 16.0	37.9 ± 11.1[a] (135)	2.0 ± 4.7	3.1 ± 3.7[b] (50)

Note. Data represent averages of five to six determinations, ± SD. The number in parentheses indicates the increment produced by dexamethasone as percentage of control. One unit of PLIP inhibits 1 unit of Naja naja naja snake venom phospholipase A_2 activity. One unit of enzyme hydrolyzes 1 μmole of L-α-phosphatidylcholine to L-α-lysophosphatidylcholine and a fatty acid per min at pH 8.9 at 25°C.

[a] $P < 0.04$, with versus without dexamethasone by Student's t test.

[b] $P < 0.01$, B10.A versus B10.

TABLE II. INDUCTION OF PHOSPHOLIPASE A_2-INHIBITORY PROTEINS BY DEXAMETHASONE IN B10.A AND B10 EMBRYONIC PALATES

	Dexamethasone (munit/palate)			
	B10.A		B10	
PLIP mol wt	Without	With	Without	With
Total	2.51 ± 1.10	6.22 ± 1.14[a,b] (148)	2.20 ± 1.20	2.90 ± 0.93 (32)
55,000	2.51 ± 1.10	6.22 ± 1.14[a,b] (148)	10.0 ± 13.0[c]	12.0 ± 9.0 (20)
28,000	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
15,000	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Note. Data represent averages of five to six determinations, ± SD. The number in parentheses indicates the increment produced by dexamethasone as percentage of control.

[a] $P < 0.01$, without versus with dexamethasone by student's t test.

[b] $P < 0.02$, B10.A versus B10.

PLIP, and inhibition of prostaglandin biosynthesis and the induction of cleft palate.

The induction of phospholipase A_2-inhibitory proteins by dexamethasone has also been identified by three other groups of investigators (9–12, 17, 18). One group called it macrocortin (mol wt 15,000), since it is derived from macrophages (11). The second group isolated it from rabbit neutrophils and called it lipomodulin, mol wt of 40,000 (10). The third group named it renocortin (mol wt 15,000 and 30,000) which they obtained from renomedullary interstitial cells (17). In mouse thymus and embryonic palates, we have obtained phospholipase A_2-inhibitory proteins of mol wt 55,000 in addition to the smallest molecular weight species reported by the other investigators. It has been shown that the monoclonal antibody Rm23 reacts with lipomodulin, macrocortin, and renocortin, and inhibits their biological activity (18, 19). Based on this observation these authors concluded that these proteins are not reserved only for inflammatory action. It appears from our observations that these proteins may be involved even in the teratogenic actions of glucocorticoids. Thus, this phenomenon would seem to be of a more general physiological relevance.

The present results demonstrate that the *H-2* gene complex influences both the endogenous amount of PLIP and the amount of PLIP induced by dexamethasone in thymocytes. In the embryonic palate, the *H-2*

TABLE III. INDUCTION OF PHOSPHOLIPASE A_2-INHIBITORY PROTEINS BY DEXAMETHASONE IN AWY AND ABY EMBRYONIC PALATES

	Dexamethasone (munit/palate)			
	AWY		ABY	
PLIP mol wt	Without	With	Without	With
Total	3.40 ± 0.52	7.10 ± 1.10[a,b] (109)	2.90 ± 0.61	5.30 ± 1.42[a] (83)
55,000	2.20 ± 0.76	4.20 ± 0.87[a,b] (91)	1.80 ± 0.37	2.72 ± 0.67[a] (20)
28,000	0.62 ± 0.39	1.07 ± 0.57 (73)	0.45 ± 0.29	1.02 ± 0.49 (127)
15,000	0.58 ± 0.22	2.38 ± 1.12[a] (310)	0.65 ± 0.32	1.52 ± 1.45 (134)

Note. Data represent averages of five to six determinations, ± SD. The number in parentheses indicates the increment produced by dexamethasone as percentage of control.

[a] $P < 0.01$, without versus with dexamethasone by Student's t test.

[b] $P < 0.02$, AWY versus ABY.

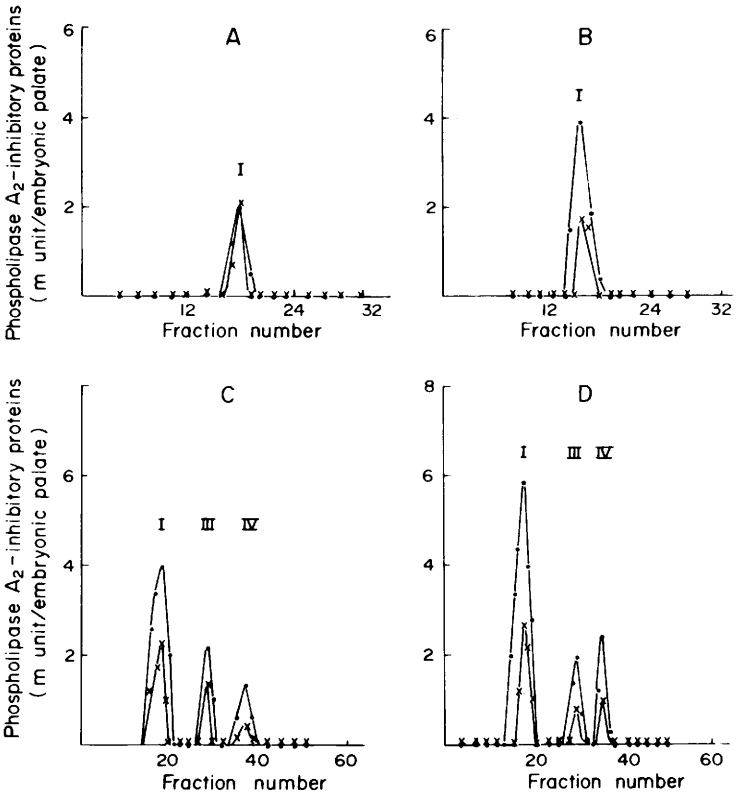

FIG. 2. Analysis of PLIP prepared from embryonic palates by Sephadex G-100 in the presence (●) and in the absence of dexamethasone (×) in B10 (A), B10.A (B), A.BY (C), and AWy (D) is shown.

gene complex influences only the amount induced by dexamethasone. This may be due to the fact that the endogenous level of PLIP in the embryonic palate is almost at the level of detectability.

Recently, Hirata *et al.* have presented some immunological evidence for the identification of lipomodulin as a product of the I-J region of the *H-2* complex (20, 21). They have shown that three molecular species (34,000, 24,000, and 16,000 Da) of [^{35}S]methionine-labeled lipomodulin are found in the im-

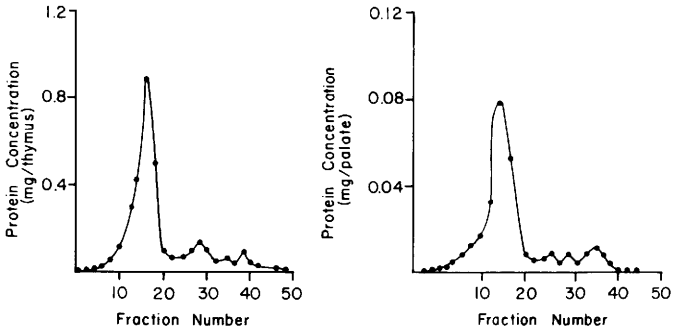

FIG. 3. Protein concentration in various fractions (determined from 280 absorption) Sephadex G-75 column chromatography of B10 thymus and B10 embryonic palates.

munoprecipitates by antilipomodulin antibody; and anti-I-J antibody precipitates the 34,000 and 24,000 Da peptides. Antilipomodulin antibody clears all species immunoreactive with anti-I-J antibody, but medium treated with anti-I-J antibody still contains a 16,000-Da peptide that reacts with antilipomodulin antibody. These results suggest, but do not necessarily prove, that antilipomodulin and anti-I-J antibodies recognize different domains of the same molecules. However, it should be noted that anti-I-J antibody has a haplotype specificity, whereas the antilipomodulin antibody is of much broader specificity. Using ^{32}P-labeled thymocytes Hirata *et al.* have shown in a time-course study on the release of ^{32}P-labeled lipomodulin that the two small peptides of 24,000 and 16,000 Da derive from a 34,000 Da peptide (21). However, Steinmetz and his associates (23) have recently cloned and characterized a stretch of DNA from the I region of the *H-2* complex. They showed that this region had no detectable I-J locus between the I-A and I-E region, suggesting that the I-J polypeptides are probably not encoded there (23). Further studies are needed to resolve this paradox. However, our results demonstrate that the level of PLIP-induced by glucocorticoids is influenced by *H-2*.

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