Distribution of the integral plasma membrane glycoprotein CE9 (MRC OX-47) among rat tissues and its induction by diverse stimuli of metabolic activation

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We have compared the levels of the integral plasma membrane glycoprotein CE9 (MRC OX-47) in different tissues of the rat and have ascertained that the levels of CE9 protein and mRNA in selected tissues and cells exhibit moderate increases in response to diverse stimuli of metabolic activation. When normalized on the basis of total protein, the level of CE9 detected in the different tissues was found to vary over a 50-fold range. In addition, the apparent molecular mass of CE9 was observed to vary from 40 kDa to 68 kDa as a consequence of tissue-specific glycosylation. The highest level of CE9 was detected in brown adipose tissue, where the protein was found to be localized to the plasma membranes of the adipocytes. The metabolic activation of brown adipose tissue that occurs upon exposure of rats to the cold was found to be accompanied by 3.0 ± 0.4 -fold and 1.7 ± 0.2 -fold increases in the levels of CE9 mRNA and protein respectively. An intermediate level of CE9 was detected in the liver,

where the protein is known to be expressed within the basolateral domain of the hepatocyte plasma membrane. The metabolic activation of hepatocytes that occurs upon administration of thyroid hormone to euthyroid rats was found to be accompanied by 2.2 ± 0.3 -fold and 1.9 ± 0.3 -fold increases in the levels of CE9 mRNA and protein respectively. A low level of CE9 was detected in the lymphoid organs, such as thymus and spleen. The metabolic activation of isolated rat splenocytes that occurs upon concanavalin A-mediated blast transformation in culture was found to be accompanied by 2.1 ± 0.2 -fold and 1.6 ± 0.2 -fold increases in the levels of CE9 mRNA and protein respectively. On the basis of these and other observations, we suggest that the level, and possibly also the localization, of the integral plasma membrane glycoprotein CE9 may be correlated in a positive fashion with metabolic activity in a diverse array of cell types.

INTRODUCTION

We have been characterizing an integral plasma membrane glycoprotein called CE9. Identified originally using monoclonal antibodies [1–3], CE9 has proved to be compartmentalized to specialized plasma membrane domains on polarized cells as diverse as epithelial cells and spermatozoa. To date, we have characterized CE9 most extensively in two tissues of the rat: (i) in the liver, where CE9 is compartmentalized to the basolateral domain of the hepatocyte plasma membrane [1–4] and is induced in response to dietary peroxisome proliferators [5]; and (ii) in the male reproductive system, where CE9 is compartmentalized to the posterior-tail plasma membrane domain of the forming spermatozoon during spermiogenesis [6–9] and experiences endo-lysosomal processing within its extracellular domain as a prelude to its redistribution from the posterior-tail to the anterior-tail plasma membrane domain during the post-testicular maturation of spermatozoa in the epididymis [6–8].

Although the function of CE9 remains to be established, we have recently determined the sequence of the protein and have found it to be a type-Ia transmembrane glycoprotein member of the immunoglobulin superfamily [7]. The sequence of CE9 proved to be identical to that of the MRC OX-47 antigen detected on rat T lymphocytes [10] and to show significant sequence similarity to putative homologues, also of unknown function, identified previously using either monoclonal antibodies or lectins in the mouse (84% identical to mouse gp42 or basigin [11,12]), human (58% identical to human basigin and M6 [13,14]) and chicken (49% identical to the chicken HT7 antigen, neurothelin or 5A11 antigen [15–17]). In some cases, the tissue distributions of these

immunoglobulin superfamily members have been investigated by immunohistochemistry using the available monoclonal antibodies, and the resulting data regarding protein localization have been used to help infer function. For example, the chicken homologue has been localized to the endothelial cells in the brain, but not to those in non-neural tissues, thus leading to speculation that it might function as a receptor or transporter that operates at the blood–brain barrier [15,16]. Likewise, the detection of the MRC OX-47 monoclonal epitope in greater numbers on the surfaces of rat T lymphocytes following concanavalin A-mediated blast transformation in cell culture [18] has resulted in the designation of the MRC OX-47 antigen as a T cell activation antigen and has fuelled speculation that the protein might serve as a lymphokine receptor [10].

In the course of our characterization of CE9, we have had occasion to generate affinity-purified rabbit polyclonal antibodies directed against the polypeptide backbone of the protein [2–9,19]. These antibodies have permitted us to quantify the levels of CE9 in rat tissues with a high degree of reliability. Using these antibodies and a corresponding CE9 cDNA, we have made some unexpected observations regarding the distribution of CE9 among rat tissues and have identified additional stimuli which can alter the levels of CE9 protein and mRNA.

MATERIALS AND METHODS

Materials

The following materials were obtained from the designated sources: male Fischer F344 rats (200–225 g; Charles River

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Breeding Laboratories, Wilmington, MA, U.S.A.); [α - 32 P]ATP, [γ - 32 P]ATP and Na 125 I (Amersham Corp., Arlington Heights, IL, U.S.A.); rhodamine-labelled goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, U.S.A.); concanavalin A and 3,5,3'-tri-iodo-L-thyronine (thyroid hormone) (Sigma Chemical Co., St. Louis, MO, U.S.A.); and human 28 S ribosomal RNA oligonucleotide (CLONTECH Laboratories, Inc., Palo Alto, CA, U.S.A.). Clone 3-1 was used for the CE9 cDNA [7]. The cDNAs encoding rat cytochrome *c* and cytochrome oxidase subunits III and IV were kindly provided by Dr. Richard Scarpulla (this department). The cDNA encoding rat albumin was kindly provided by Dr. Janardan K. Reddy (Department of Pathology, this institution). Polyclonal antibodies directed against the rat plasma membrane proteins CE9, HA 4, dipeptidyl peptidase IV and HA 321 were affinity purified from immune rabbit sera [5,19].

Animals, tissues and cells

Rats were maintained on a 14-h light/10-h dark cycle and given free access to rat chow and water for 1 week before being used in experiments. For cold exposure, rats were caged separately and moved to a 4 °C room for 2 weeks [20]. During the second week, the rats were also exposed to -20 °C for 1-h periods twice per day [20]. For thyroid hormone treatment, rats were injected intraperitoneally with 60 µg/100 g body weight 3,5,3'-tri-iodo-L-thyronine (0.2 mg/ml stock in 1 mM NaOH, 0.15 M NaCl) daily for up to 8 days [21,22]. Rats were anaesthetized with diethyl ether and decapitated. Tissues to be analysed by Western blotting were excised and homogenized in 0.25 M sucrose, 3 mM imidazole/HCl, pH 7.4, containing protease inhibitors (1 mM PMSF and 1 µg/ml each of antipain and leupeptin), and the homogenates were stored at -90 °C [5]. For Northern blotting, tissues were excised and stored in liquid nitrogen. Unless specified otherwise, the whole organ was taken for analysis. For small intestine and liver, a 5 cm segment of duodenum and the largest liver lobe were used following perfusion with ice-cold saline. For skeletal muscle, rectus femoris muscles of the hindlimbs were pooled. Whole blood (approx. 3 ml/rat) was collected from the neck following decapitation. Brown and white adipose tissues were collected from interscapular and epididymal sites respectively.

Spleen cells were isolated by pressing the spleens of six rats through a wire screen into Hank's balanced salt solution. The cells were treated with 0.83 % (w/v) NH $_4$ Cl, 17 mM Tris/HCl, pH 7.2, to lyse contaminating erythrocytes, washed three times in Hank's balanced salt solution, and resuspended in RPMI 1640 medium containing 5 % (v/v) fetal bovine serum, 1 mM sodium pyruvate, 30 µM 2-mercaptoethanol, 12 mM Hepes, 1 % Fungizone, 100 units/ml penicillin G (sodium salt) and 100 µg/ml streptomycin sulphate [23]. A total of 3×10^7 cells were incubated in 100 mm dishes at 37 °C in an atmosphere containing 5 % CO $_2$ in the presence or absence of 5 µg/ml concanavalin A [18]. After 48 h in culture, the cells were collected by low-speed centrifugation and homogenized either in guanidine thiocyanate for isolation of RNA or in 0.25 M sucrose, 3 mM imidazole/HCl, pH 7.4, containing protease inhibitors, for the analysis of proteins.

Northern and Western blotting

Total cellular RNA was prepared by homogenization in guanidine thiocyanate followed by centrifugation in CsCl [24]. For Northern blotting, 10–15 µg of RNA was electrophoresed in formaldehyde denaturing agarose gels, transferred to nitro-

cellulose by capillary action and probed with randomly primed 32 P-labelled cDNA [25]. The 28 S ribosomal RNA oligonucleotide was radiolabelled at its 5' end using [γ - 32 P]ATP [25] and used to reprobe the same Northern blots. SDS gel samples were prepared from tissue homogenates, resolved by electrophoresis in SDS/polyacrylamide gels, transferred to nitrocellulose and probed with affinity-purified rabbit polyclonal antibodies followed by 125 I-labelled Protein A [4–9]. The affinity-purified rabbit polyclonal anti-CE9 antibody has been demonstrated to react with the polypeptide backbone of the CE9 protein and not with its glycans [6,7,9]. CE9 was immunoprecipitated from Triton X-100/n-octyl- β -D-glucopyranoside extracts of tissue homogenates prepared in the presence of protease inhibitors using either monoclonal or polyclonal antibodies conjugated to Sepharose 4B [3–6,9]. Immunoprecipitated CE9 was deglycosylated chemically using a combination of trifluoromethanesulphonic acid and anisole [3] before being analysed on Western blots.

mRNA and plasma membrane protein levels were quantified by scanning laser densitometric analysis (Ultrosan XL; Pharmacia LKB Biotechnology, Inc., Piscataway, NJ, U.S.A.) of the autoradiograms [19]. The relative concentrations of CE9 and other plasma membrane proteins were calculated by normalizing on the basis of total homogenate protein as determined by a modified Lowry protein assay [26]. The relative concentration of CE9 mRNA was calculated by normalizing on the basis of 28 S ribosomal RNA as determined by reprobating the same Northern blots, but the results were similar if the data were normalized on the basis of total RNA concentration estimated using A_{260} . The relative concentrations of plasma membrane proteins and mRNAs are reported as means $\pm$ S.D. for the designated number of animals or cell isolations. Significance was estimated through the application of Student's *t* test.

Immunofluorescence

Rat tissues were fixed by perfusion (liver) or immersion (brown adipose tissue) in ice-cold 2 % paraformaldehyde/lysine/periodate fixative for 30 min and infiltrated with a buffered solution of sucrose and polyvinylpyrrolidone overnight at 4 °C [5]. Sections of 1 µm thickness were cut at -80 °C using a Reichert-Jung (Vienna, Austria) Ultracut E ultramicrotome equipped with an FC-4D cryo attachment, collected on poly(L-lysine)-coated slides and labelled sequentially with affinity-purified antibody or preimmune IgG and rhodamine-labelled goat anti-rabbit IgG in the presence of 0.25 % (v/v) Triton X-100 [5].

RESULTS

Western blotting was used to compare the forms of CE9 detected in different organs and tissues of the rat. Although CE9 appears to be encoded by a single gene and mRNA [7], we noted considerable heterogeneity among the CE9 polypeptides detected within the different rat tissues. Depending upon the tissue, immunologically cross-reactive proteins were found to migrate within the range 40–68 kDa in SDS/polyacrylamide gels, often as broad bands or partially resolved multiplets (Figure 1). These proteins most likely represented *bona fide* CE9, because each could be immunoprecipitated, whether using polyclonal or monoclonal anti-CE9 antibodies (Figure 2 and results not shown). This applied even to the relatively minor ~40 kDa bands that migrated significantly faster than the bulk of the immunoreactive material in samples prepared from homogenates of heart and liver (Figures 1 and 2); 40 kDa is the apparent

Figure 1 Different amounts and forms of the CE9 protein detected in rat tissues

Organs and tissues were removed from control rats, and the CE9 protein was revealed by Western blotting using affinity-purified rabbit polyclonal anti-CE9 antibody and 125 I-Protein A. The lanes were loaded with approximately equivalent amounts of total protein, with the exception of the lane containing the sample of thymus homogenate, which was loaded with approximately three times the standard amount of total protein. BA, brown adipose tissue; TE, testis; KI, kidney; HE, heart; LI, liver; BR, brain; SI, small intestine; WA, white adipose tissue; TH, thymus; SP, spleen.

Figure 2 Chemical deglycosylation of tissue-specific forms of CE9 to yield a common 32 kDa polypeptide

CE9 was isolated from non-ionic detergent extracts of the designated tissues by immunoprecipitation using rabbit polyclonal anti-CE9 IgG conjugated to Sepharose 4B and analysed by Western blotting without (a) and with (b) prior chemical deglycosylation using trifluoromethanesulphonic acid and anisole. SM, skeletal muscle; LI, liver; SI, small intestine; KI, kidney; TE, testis; BR, brain; HE, heart; SP, spleen.

molecular mass of the high-mannose precursor of CE9 in rat liver [5]. The only exception was the minor ~ 68 kDa band found in the sample prepared from brain homogenate (Figure 1); this protein was not immunoprecipitated by either polyclonal or monoclonal antibodies (Figure 2 and results not shown) and was, therefore, ignored in our quantification (see below). On the basis of a difference in the number of asparagine-linked glycans detected when comparing the forms of CE9 isolated from liver and testis [7], we suspected that the heterogeneity in apparent molecular mass detected among the forms of CE9 observed in the different rat tissues was the result of differential deglycosylation of the same polypeptide backbone. In support of this notion, the different forms of CE9 were all found to co-migrate at the expected apparent molecular mass of ~ 32 kDa [7] following chemical deglycosylation with trifluoromethanesulphonic acid and anisole. This effect is illustrated in Figure 2 for the CE9 polypeptides isolated by immunoprecipitation from eight different rat tissues.

Table 1 Quantification of CE9 in rat tissues

The relative level of CE9 protein detected in each organ or tissue of control rats was determined by Western blotting after normalization on the basis of total protein. The results are tabulated as means $\pm$ S.D. ($n = 3$), with the relative level of CE9 found in brown adipose tissue assigned a value of 100.

Organ or tissue	Relative level of CE9
Brown adipose tissue	100 $\pm$ 1
Testes	90 $\pm$ 3
Kidney	52 $\pm$ 2
Heart	48 $\pm$ 1
Liver	44 $\pm$ 1
Brain	41 $\pm$ 2
Small intestine	38 $\pm$ 1
White adipose tissue	30 $\pm$ 3
Skeletal muscle	19 $\pm$ 1
Thymus	15 $\pm$ 1
Spleen	10 $\pm$ 1
Blood	2 $\pm$ 1

We next used Western blotting to compare the amounts of CE9 in the different organs and tissues of the rat. The Western blotting was performed under conditions that were verified to give a linear or near-linear response as a function of input over the concentration range of interest through the construction of standard curves [19]. When normalized on the basis of total protein, the amount of CE9 was found to vary over a 50-fold range (Table 1). The highest levels of CE9 were found in brown adipose tissue and testes. Intermediate levels were found in kidney, heart, liver, brain, small intestine, white adipose tissue and skeletal muscle. The lymphoid tissues, i.e. thymus and spleen, and whole blood were found to contain the lowest levels of CE9.

Because of the precedent of the induction of the MRC OX-47 monoclonal epitope and the corresponding mRNA during concanavalin A-mediated T lymphocyte activation in culture [10,18], we wondered whether CE9 might also be induced in those cases in which cells are caused to undergo metabolic activation *in vivo*. For these experiments we used Northern and Western blotting to quantify the levels of CE9 mRNA and protein respectively. Both types of blotting were performed under conditions that were verified to give a linear or near-linear response as a function of input over the concentration range of interest through the construction of standard curves (e.g. see [19]). mRNA levels were normalized on the basis of 28 S rRNA, and protein levels were normalized on the basis of total tissue protein.

The most concentrated source of CE9, brown adipose tissue (Table 1), is responsible for non-shivering thermogenesis in the rat and is known to become metabolically activated upon exposure to the cold [27,28]. When rats were exposed to the cold for 2 weeks [20], the level of CE9 mRNA in intrascapular brown adipose tissue was found to be increased 3.0 ± 0.4 -fold ($n = 3$; $P < 0.005$) (Figure 3a). This cold-induced increase in the level of CE9 mRNA was found to be mirrored by a 1.7 ± 0.2 -fold ($n = 3$; $P < 0.005$) increase in the level of CE9 protein (Figure 3a). These increases most likely reflected increased accumulation of CE9 mRNA and protein by the adipocytes of brown fat and not other cell types, because CE9 appeared to be concentrated at the surfaces of the adipocytes, both with (Figure 4a) and without (results not shown) cold exposure.

Figure 3 Induction of CE9 mRNA and protein in brown adipose tissue by cold exposure (a), in liver by injection of thyroid hormone (b), and in cultured splenocytes by concanavalin A (c)

Rats were either exposed to the cold or injected with thyroid hormone, or rat splenocytes were cultured in the presence of concanavalin A, as described in Materials and methods section (+). The control rats were either housed at room temperature or injected with vehicle alone, or their splenocytes were cultured in the absence of concanavalin A, respectively (-). 28 S rRNA and CE9 mRNA were detected by Northern blotting of duplicate samples containing 10–15 μ g of total RNA. CE9 protein (CE9 Prot.) was detected by Western blotting of samples of tissue/cell homogenate containing equivalent amounts of total protein.

We next turned to the consideration of an organ that was found to express an intermediate level of CE9, the liver (Table 1). Hepatic parenchymal cells, the hepatocytes, are known to become metabolically activated in response to the administration of thyroid hormone [29,30]. Daily intraperitoneal injections of thyroid hormone (60 μ g/100 g body weight) administered to euthyroid rats for 8 days [21,22] were found to increase the level of CE9 mRNA 2.2 ± 0.3 -fold ($n = 3$; $P < 0.005$) over that observed in the control rats, which received injections of vehicle alone (Figure 3b). Although intraperitoneal injections of thyroid hormone are known to increase the level of total poly(A)⁺-containing mRNA by as much as 60–75% in hypothyroid rats, no such generalized increases are observed when thyroid hormone is administered to euthyroid rats [31,32]. The thyroid hormone-induced increase in the level of hepatic CE9 mRNA was found to be mirrored by a 1.9 ± 0.3 -fold ($n = 3$; $P < 0.005$) increase in the level of hepatic CE9 protein (Figure 3b). When quantified by Western blotting on the same blots using the corresponding affinity-purified rabbit polyclonal antibodies, the levels of three other hepatocyte integral plasma membrane glycoproteins (HA 4, HA 321 and dipeptidyl peptidase IV [5,19]) were found not to change in response to the administration of thyroid hormone (results not shown). Thus the increase in the level of hepatic CE9 protein detected in the livers of the thyroid hormone-treated rats was found to be the same whether the data were normalized on

Figure 4 Immunolocalization of CE9 to the plasma membranes of brown adipocytes following cold exposure (a) and to the plasma membranes of hepatocytes in thyroid hormone-treated rats (b)

Frozen semi-thin sections were labelled sequentially with affinity-purified rabbit polyclonal anti-CE9 antibody and rhodamine-labelled goat anti-rabbit IgG.

the basis of total liver protein or to the levels of any one of these three other hepatocyte integral plasma membrane glycoproteins. The thyroid hormone-induced increases most likely reflected increased accumulation of CE9 mRNA and protein by hepatocytes and not other hepatic cell types, because CE9 appeared to be concentrated at the basolateral domain of the hepatocyte plasma membrane, either with (Figure 4b) or without (results not shown) injection with thyroid hormone.

When we examined the time course of the induction of CE9 mRNA in the liver by thyroid hormone, we found the response to be relatively slow, with 2 days being required to observe significant increases over control levels (Table 2). Both the rate and magnitude of the induction of CE9 mRNA in liver in response to thyroid hormone were less than those observed for cytochrome *c* mRNA [33], but were similar to those observed for the mRNAs which encode two other key components of the mitochondrial respiratory chain, cytochrome oxidase subunits III and IV (Table 2). The rate and magnitude of the induction that we observed for cytochrome *c* mRNA were similar to those reported by Scarpulla et al. [33], suggesting that thyroid hormone indeed elicited the expected metabolic activation of hepatocytes in our experiments. Not all hepatocyte mRNAs were found to increase in response to the administration of thyroid hormone, however; for example, the level of the mRNA encoding albumin was found to decrease slightly during this same interval (Table 2).

Finally, we extended the analysis to the spleen, a source which was found to contain some of the lowest levels of CE9 (Table 1). To bring about the metabolic activation of spleen cells, we cultured them for 48 h in the presence of 5 μ g/ml concanavalin A to elicit blast transformation [35]. The splenocytes cultured in the presence of concanavalin A were found to have 2.1 ± 0.2 -fold

Table 2 Time course of the induction of liver mRNAs in response to thyroid hormone

Rats were injected with either thyroid hormone or vehicle alone as described in the Materials and methods section. Total liver RNA was isolated at the designated times, and the relative levels of the different mRNAs were determined by Northern blotting after normalization on the basis of 28 S rRNA. The results are tabulated as means $\pm$ S.D. ($n = 3$), with the relative level of a given mRNA found in the vehicle control assigned a value of 1.0. *Significantly greater than vehicle control; $P < 0.005$.

Time (h)	Relative level of mRNA				
	Cytochrome <i>c</i>	CE9	Cytochrome oxidase IV	Cytochrome oxidase III	Albumin
8	1.6 $\pm$ 0.1*	1.0 $\pm$ 0.1	1.2 $\pm$ 0.1	1.0 $\pm$ 0.1	1.0 $\pm$ 0.1
24	2.4 $\pm$ 0.3*	1.3 $\pm$ 0.1	1.5 $\pm$ 0.2	1.1 $\pm$ 0.2	0.8 $\pm$ 0.1
48	3.6 $\pm$ 0.3*	2.3 $\pm$ 0.2*	2.2 $\pm$ 0.4*	1.7 $\pm$ 0.1*	0.8 $\pm$ 0.1
72	3.4 $\pm$ 0.3*	2.1 $\pm$ 0.2*	2.5 $\pm$ 0.1*	1.8 $\pm$ 0.1*	0.8 $\pm$ 0.1
96	2.8 $\pm$ 0.2*	2.1 $\pm$ 0.2*	2.1 $\pm$ 0.1*	2.0 $\pm$ 0.3*	0.7 $\pm$ 0.1

($n = 3$; $P < 0.01$) and 1.6 $\pm$ 0.2-fold ($n = 3$; $P < 0.01$) higher levels of CE9 mRNA and protein respectively than control splenocytes cultured in the absence of concanavalin A (Figure 3c).

DISCUSSION

CE9 was identified independently as a basolateral domain-specific plasma membrane protein of rat hepatocytes [1–3] and as the MRC OX-47 antigen, a monoclonal epitope detected in larger amounts on rat T cells following concanavalin A-induced blast transformation *in vitro* [10,18]. Our data indicate that CE9 is by no means confined to hepatocytes or T cells, but instead appears to be expressed to some extent in virtually every tissue of the rat. This suggests that CE9 performs a function that is required by most cells, not just a small subset. In fact, of all of the tissues examined, we found the lymphoid tissues to contain some of the lowest levels of CE9 (Table 1). Although Fossum et al. [10] also presented evidence for a broad distribution of the MRC OX-47 monoclonal epitope among rat tissues, their analysis relied exclusively on the use of monoclonal antibody immunoperoxidase histochemistry. Since their monoclonal antibody failed to react by immunoprecipitation or Western blotting [10,18], there was no reliable way for them to demonstrate the specificity of their monoclonal antibody for the MRC OX-47 polypeptide in the different tissues, nor was it possible for them to obtain reliable estimates of the relative levels of the MRC OX-47 antigen among rat tissues.

We found that the induction of CE9 is not a response that is limited to the activation of T cells in culture [10,18], but instead appears to be part of a generalized response to stimuli that bring about an increase in cellular metabolic rate. Whether assayed at the level of the mRNA or protein, we found the magnitude of the induction which accompanies metabolic activation to be moderate, typically about 2-fold. This held for activating stimuli as diverse as cold exposure (brown adipocytes) and thyroid hormone (hepatocytes) *in vivo* and concavalin A-mediated cross-linking of cell surface receptors in culture (splenocytes). At first glance, the increases in the levels of CE9 protein noted in our experiments with culture splenocytes appear to be significantly less than the 5.2-fold increase in cell surface MRC OX-47 monoclonal epitopes reported using fluorescence-activated cell sorting to compare T blasts generated by concanavalin A in cell culture with the corresponding thymocyte controls [18]. However, here again, we must point out that the experiments of Paterson et al. [18] involved the use of a monoclonal antibody of indeterminate specificity. Furthermore, there is no reason to assume that at

least part of the additional MRC OX-47 monoclonal epitopes detected at the surfaces of the T blasts did not arise through the unmasking or externalization of molecules that pre-existed intracellularly. Using confocal immunofluorescence microscopy, we have noted that some cells do indeed maintain significant pools of CE9 intracellularly within elements of the secretory pathway [9].

Although a 2-fold increase in the levels of CE9 mRNA and protein might seem small, this is precisely the magnitude of the effect that stimuli such as thyroid hormone or cold exposure have on critical metabolic parameters such as oxygen consumption, caloric intake and fat content [22,27]. Furthermore, these far-reaching metabolic consequences are known to be accompanied by comparably moderate increases in the expression of a number of important cellular proteins and their mRNAs in the relevant target tissues, e.g., Na⁺, K⁺-ATPases [21,34], glucose transporters [35], lipogenic enzymes [22,36] and the proteins which comprise the respiratory chain of mitochondria [33,37,38]. Increments in the levels of expression of the various subunits of cytochrome oxidase have been correlated directly with metabolic activity or workload in a variety of contexts (e.g. see [39,40]). The ability of thyroid hormone to induce CE9 in hepatocytes might help to explain our previous observation that CE9 is induced $\sim$ 2-fold by dietary peroxisome proliferators [5], xenobiotic amphipathic carboxylates which are known to exert a number of thyromimetic effects [41]. It is not clear whether the increases in the level of CE9 mRNA that we observe in the various models of cellular activation are due to increased transcription of the CE9 gene or stabilization of the CE9 mRNA. Unfortunately, the relatively low magnitude of the effect has thus far made it impossible for us to distinguish between these two possibilities experimentally. What we can say, however, is that the response to thyroid hormone is sufficiently delayed to rank as a late or adaptive effect. That such late effects are often attributed to supplemental mitochondrial biogenesis [42] may help to explain the correlations that we observe between the induction of CE9 mRNA and the mRNAs encoding the subunits of cytochrome oxidase. The possible relationship between CE9 and mitochondria may, in turn, help to explain why brown adipose tissue and cardiac muscle contain more CE9 than white adipose and skeletal muscle respectively, and why CE9 is positioned near the mitochondria in the plasma membrane of the basolateral surface infoldings of certain transporting epithelial cells ([10]; C. L. Nehme, B. E. Fayos and J. R. Bartles, unpublished work) and the midpiece of the mature spermatozoon [6,8]. In this context, it has not escaped our attention that CE9 redistributes to come into alignment over the energy-generating mitochondria of the spermatozoal mid-

piece at the time during post-testicular maturation when these cells acquire the ability to move in a forward direction and to fertilize [43–45].

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