POSTTRANSCRIPTIONAL REGULATION OF ICAM-1 GENE EXPRESSION

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ABSTRACT

Cell-cell adhesion is critical for the generation of effective immune responses and is dependent upon the expression of a variety of cell surface receptors. Intercellular adhesion molecule-1 (ICAM-1; CD54) is an inducible cell surface glycoprotein expressed at a low level on a wide range of cell types. Although its expression is dramatically increased at sites of inflammation, providing important means of regulating cell-cell interactions and thereby inflammatory responses, the intracellular regulatory elements and signaling pathways underlying the inducible expression of ICAM-1 by proinflammatory cytokines were previously largely unknown. In this thesis, a novel posttranscriptional regulation of ICAM-1 gene expression by two proinflammatory mediators, interferon-γ (IFN-γ) and phorbol myristate acetate (PMA), and the possible role of serine/threonine (ser/thr) phosphorylation pathway in cycloheximide-induced ICAM-1 message stabilization were investigated. The results show that (1) constitutively expressed ICAM-1 mRNA has a short half-life; (2) IFN-γ and PMA induce the accumulation of ICAM-1 message, at least in part, by stabilizing the mRNA; (3) the IFN-γ-responsive element(s) is located within the protein coding region encoding the cytoplasmic domain; (4) the PMA-responsive elements lie within the 3' untranslated region (UTR) and may even be the AUUUA multimers; (5) cycloheximide, a potent eukaryotic protein synthesis inhibitor that superinduces the expression of many genes by preventing the degradation of otherwise labile mRNAs, also induces the level of ICAM-1 mRNA by message stabilization; (6) the stabilizing effect of cycloheximide does not depend on the 3'UTR containing the AUUUA sequences; (7) the stabilization of ICAM-1 mRNA by cycloheximide is independent of its translational inhibition; and (8) the ser/thr phosphorylation pathway seems to play a crucial role in the cycloheximide-induced stabilization of ICAM-1 message. These results demonstrate the existence of distinct destabilizing elements throughout the ICAM-1 message that are responsive to the actions of various proinflammatory cytokines, and underscore the importance of posttranscriptional regulation of ICAM-1 expression during an inflammatory response.

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ABBREVIATIONS

aa	amino acid
ActD	actinomycin D
APC	antigen-presenting cell
ARE	AU-rich element
bp	base pair
BSA	bovine serum albumin
cAMP	cyclic AMP; adenosine 3', 5'-monophosphate
CD	cluster of differentiation
cDNA	complementary DNA
CHX	cycloheximide
CMV	cytomegalovirus
cpm	counts per minute
DMEM	Dulbecco's modified minimum essential media
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
ECM	extracellular matrix
EDTA	ethylenediamine tetraacetic acid
EtBr	ethidium bromide
FACS	flurorescence activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
fMLP	f-Met-Leu-Phe
g	gram
GlyCAM-1	glycosylation-dependent cell adhesion molecule-1
GM-CSF	granulocyte/macrophage colony-stimulating factor

HBSS	Hanks' balanced salt solution
h	hour
HUVEC	human umbilical vein endothelial cell
ICAM	intercellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IRE	iron-responsive element
IRE-BP	IRE-binding protein
kb	kilobase
kD	kilodalton
LAD	leukocyte adhesion deficiency
LFA	lymphocyte function-associated antigen
LPS	lipopolysaccharide
mAb	monoclonal antibody
Mac-1	macrophage antigen-1
MAdCAM-1	mucosal addressin cell adhesion molecule-1
MAG	myelin-associated glycoprotein
MHC	major histocompatibility complex
min	minute
MLR	mixed lymphocyte reaction
mRNA	messenger RNA
NCAM	neural cell adhesion molecule
NK	natural killer
NP-40	nonidet P-40
nt	nucleotide
ORF	open reading frame

PAB	poly(A)-binding protein
PAGE	polyacrylamide gel electrophoresis
PAN	poly(A) nuclease
PBL	peripheral blood lymphocytes
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHA	phytohaemagglutinin
PKA	protein kinase A
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PSGL-1	P-selectin glycoprotein ligand-1
RBC	red blood cell; erytrocytes
RGD	Arg-Gly-Asp
RNA	ribonucleic acid
rRNA	ribosomal RNA
SDS	sodium dodecyl sulphate
sICAM	soluble ICAM
SSC	saline sodium citrate buffer
SSPE	saline sodium phosphate EDTA buffer
TCR	T-cell receptor
TE	Tris EDTA buffer
TNE	Tris sodium chloride EDTA buffer
TNF	tumor necrosis factor
TRE	TPA-responsive element
tRNA	transfer RNA
UTR	untranslated region
VCAM	vascular cell adhesion molecule

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For my mother and father.

I

INTRODUCTION

Adhesive interactions of cells with each other and with the extracellular matrix (ECM) are features of many processes in multicellular organisms and also appear to play important roles in the development and functional responses of immune cells. During development of the embryo, cell adhesion proteins impart position-specific information that guide cell migration, localization, and the transfer of information between cells. As the cells are triggered to differentiate to form specific components of the various tissues and organs, adhesion proteins help to maintain the organization and integrity of the body. The immune system is comprised of a variety of circulating cells which utilize highly specialized cellular recognition mechanisms. The function of the immune system is to distinguish self from the nonself and to eliminate the latter. This involves the continuous circulation of leukocytes through the blood and lymphatic systems in surveillance of foreign materials. When such antigens (or epitopes) are detected, the circulating leukocytes migrate to the site and mount an immune or inflammatory reaction. Three major families of cell adhesion molecules (Table 1), the selectin family, the integrin family, and the immunoglobulin (Ig) superfamily, have been found to play important roles in lymphocyte recirculation, migration of leukocytes into inflamed tissues, and cell-cell interactions involved in the generation of immune responses. This thesis focusses on one of these adhesion molecules, the intercellular adhesion molecule-1 (ICAM-1).

Table 1. Selected members of the three major cell adhesion families[1].

Ig Superfamily	Distribution	Ligands
ICAM-1 (CD54)	lymphocytes, neutrophils, thymocytes, monocytes, dendritic cells, endothelium, fibroblasts, keratinocytes, chondrocytes, epithelium	LFA-1, Mac-1, CD43, fibrinogen
ICAM-2 (CD102)	endothelium, subpopulation of lymphocytes, monocytes, dendritic cells	LFA-1
ICAM-3 (CD50)	lymphocytes, monocytes, granulocytes	LFA-1
Leukocyte Integrins		
LFA-1 (CD11a/CD18)	lymphocytes, monocytes, neutrophils	ICAM-1, ICAM-2, ICAM-3
Mac-1 (CD11b/CD18)	monocytes, neutrophils	ICAM-1, iC3b, fibrinogen, factor X
p150,95 (CD11c/CD18)	monocytes, neutrophils	iC3b, fibrinogen
Selectins		
L (leukocyte)-selectin (CD62L, Mel-14, LAM-1)	lymphocytes, neutrophils, thymocytes, monocytes, eosinophils, basophils, NK cells	sialyl Lewis^{x,a}, GlyCAM-1[2], CD34, MAdCAM-1
P (platelet)-selectin (CD62P, PADGEM, GMP-140)	platelets (α granules), endothelium (Weibel-Palade bodies), megakaryocytes	sialyl Lewis^{x,a}, PSGL-1
E (endothelial)-selectin (CD62E, ELAM-1)	endothelium	sialyl Lewis^{x,a}, E-selectin
Other		
CD44 (H-CAM, GP-90 Hermes)	lymphocytes, monocytes, neutrophils, epithelium, glial cells, fibroblasts, myocytes	hyaluronate, collagen, chondroitin sulfated CD44 binds fibronectin

1 from the reviews by Springer, 1994 and Carlos and Harlan, 1994.

2 abbreviations used: GlyCAM-1, glycosylation-dependent cell adhesion molecule-1; MAdCAM-1, mucosal addressin cell adhesion molecule-1; PSGL-1, P-selectin glycoprotein ligand-1.

1.1 ICAM-1

ICAM-1 (CD54) is an inducible cell surface ligand for the lymphocyte function-associated antigen-1 (LFA-1; CD11a/CD18) (Rothlein *et al.*, 1986; Marlin and Springer, 1987; Makgoba *et al.*, 1989; Staunton *et al.*, 1990) and the macrophage antigen-1 (Mac-1; CD11b/CD18) (Diamond *et al.*, 1991), as well as CD43 (Rosenstein *et al.*,1991) and fibrinogen (Languino *et al.*, 1993). This membrane protein is a single chain glycoprotein with a peptide backbone of 55 kD (Staunton *et al.*, 1988; Simmons *et al.*, 1988). Human ICAM-1 is encoded by a single-copy gene located on chromosome 19 (Katz *et al.*, 1985). The gene for murine ICAM-1 has been mapped to the proximal short arm of chromosome 9 (Ballantyne *et al.*, 1991). cDNA cloning in the human (Staunton *et al.*, 1988; Simmons *et al.*, 1988) and mouse (Horley *et al.*, 1989; Siu *et al.*, 1989) has shown that ICAM-1 is a member of the Ig superfamily. Members of the Ig superfamily contain one or more Ig domains, each composed of 90-100 amino acids (aa) arranged in two sheets of anti-parallel β-strands, which is usually stabilized by a disulphide bond at its center (Williams and Barclay, 1988; Williams, 1987). ICAM-1 consists of five Ig-like extracellular domains, and displays highest homology with two other cell adhesion molecules, neural cell adhesion molecule (NCAM) and myelin associated glycoprotein (MAG). Murine ICAM-1 has limited similarity with human ICAM-1 at either the nucleotide (65%) or protein levels (50%) (Horley *et al.*, 1989; Siu *et al.*, 1989). Amino acid substitutions in the extracellular domains have indicated that the primary binding site for LFA-1 is located in the NH_2-terminal first domain of ICAM-1 (Staunton *et al.*, 1990). Initial electron microscopy of soluble ICAM-1 suggested a hinge between the second and third domains of the extracellular region (Staunton *et al.*, 1990); however, a recent report has shown that the hinge in ICAM-1 occurs between third and fourth domains (Kirchhausen *et al.*, 1993). The location of this hinge may be germane for leukocyte adhesion to endothelium because a second ligand-binding site for a leukocyte integrin, Mac-1, has been localized to the third Ig-like domain (Diamond *et al.*, 1991).

Leukocyte adhesion to the second ligand-binding site of ICAM-1 is affected by the degree of glycosylation (Diamond *et al.*, 1991). There are eight possible sites for N-linked glycosylation in the five extracellular domains of human ICAM-1 (Staunton *et al.*, 1988; Simmons *et al.*, 1988). In contrast, murine ICAM-1 has nine potential residues for N-linked glycosylation (Horley *et al.*, 1989). The molecular weights of ICAM-1 have been found to vary between 76 and 114 kD, suggesting that there is variable posttranslational modification of ICAM-1 (Rothlein *et al.*, 1986; Dustin *et al.*, 1986; Pober *et al.*, 1986). Affinity of leukocytes for the binding site within the third Ig-like region of ICAM-1 was found to increase as the degree of glycosylation decreased (Diamond *et al.*, 1991). This observation suggested that selectivity of leukocyte adhesion via Mac-1 may be dictated in part by the posttranslational glycosylation of ICAM-1 at the tissue level.

The cytoplasmic domain of ICAM-1 consists of a 28-aa residue, highly charged sequence rich in lysine and arginine residues (Staunton *et al.*, 1988; Simmons *et al.*, 1988). The cytoplasmic domain, or an 8-aa portion of the cytoplasmic region of ICAM-1 (RQRKIKKR), has been shown to bind to the cytoskeleton of COS cells transfected with the cDNA of human ICAM-1 and to the cytoskeleton of Epstein-Barr virus (EBV)-transformed B cells (Carpen *et al.*, 1992). The binding of ICAM-1 to the cytoskeleton was found to occur through linkage with α-actinin, a cytoskeleton protein that may serve to anchor actin filaments to the cell membrane (Mimura and Asano, 1987). When expressed as a glycophosphotidylinositol-linked membrane protein, ICAM-1 was diffusely expressed on the cell surface (Carpen *et al.*, 1992). Thus, linkage with the cytoskeleton may localize ICAM-1 within regions of the endothelial cell membrane to facilitate leukocyte adherence and transmigration.

Study of LFA-1/ICAM-1 cross-species interactions has shown that human LFA-1 interacts with murine ICAM-1 but murine LFA-1 does not associate with human ICAM-1. This restriction in cross-species binding is due to the LFA-1 alpha subunit, since mouse-human

hybrids expressing human alpha chain and murine beta chain will bind human ICAM-1 (Larson and Springer, 1990).

Adhesion of ICAM-1 to these leukocyte integrins plays an essential role in a variety of immune reactions including T cell-mediated killing, natural lytic events, T-helper and B-lymphocyte responses, homotypic aggregation, antibody-dependent cytotoxicity mediated by monocytes and granulocytes, and leukocyte trafficking processes such as adherence of leukocytes to vacular endothelium and epidermal cells (Springer, 1990; Dustin and Springer, 1991; Boyd *et al.*, 1988; Carlos and Harlan, 1994).

In contrast to LFA-1, which is expressed only on leukocytes in a constitutive manner, ICAM-1 is an inducible cell surface glycoprotein expressed at a low level on a wide variety of cells, including leukocytes, vascular endothelium, fibroblasts, follicular dendritic cells, synovial cells, keratinocytes, and certain epithelial cells (Dustin *et al.*, 1986, 1988; Pober *et al.*, 1986; te Velde *et al.*, 1987; Mentzer *et al.*, 1988; Rothlein *et al.*, 1988; Dustin and Springer, 1988, 1991; Carlos and Harlan, 1994). However, ICAM-1 expression is dramatically increased at sites of inflammation (Springer, 1990; Dustin *et al.*, 1986; Dustin and Springer 1991; Rothlein *et al.*, 1988; Pober *et al.*, 1986; Carlos and Harlan, 1994), providing an important means of regulating cell-cell interactions and thereby presumably inflammatory responses. The upregulated expression of ICAM-1 on venule endothelium is thought to facilitate the adhesion and subsequent transendothelial migration of leukocytes bearing LFA-1 or Mac-1 into inflammatory tissues, as well as permitting appropriate interactions of lymphocytes with cells expressing targeted antigens (Springer, 1990; Wawryk *et al.*, 1989; Carlos and Harlan, 1994; Dustin and Springer 1991). Various proinflammatory mediators including interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), lipopolysaccharide (LPS), and interferon-γ (IFN-γ), as well as active phorbol esters, have been found to increase ICAM-1 expression on many cell types (Dustin *et al.*, 1986; Rothlein *et al.*, 1988; Carlos and Harlan, 1994) and are thought to be responsible for the induction of ICAM-1 expression at inflammatory sites *in vivo*. The function, and indeed the importance, of leukocyte adhesion

were elucidated in numerous animal experimental systems where mAbs to ICAM-1 were shown to inhibit various events associated with inflammation (Carlos and Harlan, 1994). Although the importance of leukocyte integrins was clearly illustrated in patients with LAD I syndrome (discussed below), deficiencies in ICAM-1 have not been reported in humans. However, ICAM-1 deficient mice have been generated recently and showed prominent abnormalities of inflammatory responses including impaired neutrophil emigration in response to chemical peritonitis and decreased contact hypersensitivity to 2,4-dinitrofluorobenzene (Sligh *et al.*, 1993). Furthermore, the mutant cells provided negligible stimulation in the mixed lymphocyte reaction (MLR), although they proliferated normally as responder cells (Sligh *et al.*, 1993). Similarly, Xu *et al.* (1994) observed that the homozygous ICAM-1-deficient mice have elevated numbers of circulating neutrophils and lymphocytes, as well as diminished allogeneic T cell response and delayed type hypersensitivity. These mice were also found to be resistant to septic shock either by reducing T cell activation or by diminishing neutrophil infiltration (Xu *et al.*, 1994).

In addition to its physiologic role of contributing to cell-cell adhesion in immune responses, the ability of ICAM-1 to serve as a receptor for the major serotype of rhinoviruses (Greve *et al.*, 1989; Staunton *et al.*, 1989a) has been studied. Rhinoviruses are the causative agents in 50% of common colds. Although rhinoviruses have evolved more than 100 non-crossreactive antigenic variants in an attempt to evade the immune response, 90% of them bind to ICAM-1 (Springer, 1990; Greve *et al.*, 1989; Staunton *et al.*, 1989a). More recently, soluble ICAM-1 has been shown to be a potent and specific inhibitor of major rhinovirus infection (Marlin *et al.*, 1990). Mutagenesis has indicated that there is overlap in the sequences within the first domain of ICAM-1 that interact with LFA-1 and rhinovirus. Additionally, some amino acid changes within the first domain of ICAM-1 can eliminate its ability to bind rhinovirus without affecting its ability to interact with LFA-1 (Larson and Springer, 1990).

ICAM-1 is also one of two receptors that can be subverted as a sequestration antigen for *Plasmodium falciparum*-infected erythrocytes (RBCs) (Berendt *et al.*, 1989). The primary

event in the pathogenesis of malaria is the adherence of infected RBCs to endothelium in the liver. Increased levels of cytokines have been detected in acute malaria. Thus, induction of ICAM-1 expression on endothelium resulting from cytokines may allow *P. faciparum*-infected RBCs to bind endothelial cells. Furthermore, an immunoadhesin molecule containing ICAM-1 fused to the hinge, C_H2 and C_H3 domains of the human IgG1 heavy chain has been generated and shown to inhibit the adhesion of *P. faciparum*-infected RBCs to ICAM-1-bearing surfaces, although LFA-1 interactions with ICAM-1 were not inhibited (Staunton *et al.*, 1992). The immunoadhesin also promoted phagocytosis and destruction of parasitized RBCs by human monocytes (Staunton *et al.*, 1992).

Recently, it has been demonstrated that a proportion of lymphomas including the majority of fresh Burkitt's lymphomas fail to express ICAM-1 receptor, LFA-1, on the cell surface (Clayberger *et al.*, 1987). These Burkitt's lymphoma cells are poor stimulators of both autologous and allogeneic T cell responses, suggesting that the lack of LFA-1 on these tumor cells may contribute to their failure to initiate efficient immune responses leading to their escape from immunosurveillance. Some Burkitt's lymphoma cells have been shown to be deficient in ICAM-1 and LFA-3 as well (Billaud *et al.*, 1987). ICAM-1 expression is also thought to correlate positively with the metastatic potential of malignant melanoma (Johnson *et al.*, 1989). Taken together, these observations imply that perturbation of one or a combination of adhesion molecules might be a common method by which cells acquire an ability to escape from immunosurveillance.

The importance of ICAM-1 in promoting and maintaining an effective immune response can also be appreciated by examining its leukocyte integrin receptors. The necessity for leukocytes to adhere to cellular and matrix proteins in order to function has become increasingly clear through two converging lines of evidence. First was the identification and characterization of molecules on the leukocyte surface that are responsible, in part, for multiple leukocyte adhesion events. These receptor molecules, called the CD18 family of adhesion molecules or leukocyte integrins, consist of LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18),

and p150,95 (CD11c/CD18) (for reviews, see Martz, 1982; Springer *et al.*, 1987; Springer, 1990). Each of these molecules is a heterodimer with distinct alpha subunits that associate noncovalently with a common beta subunit. These proteins are distributed in various combinations on all leukocytes. Structurally, the leukocyte adhesion molecules are members of the integrin family and specifically belong to the β_2 subgroup. Although the leukocyte adhesion molecules mediate adhesion processes, they are unlike most other integrins in that they appear to recognize ligands that, for most part, do not contain Arg-Gly-Asp (RGD) sequences and their activity is dependent on Mg^{++} rather than Ca^{++}. The function of these molecules and indeed the importance of leukocyte adhesion in the generation and maintenance of inflammation have been elucidated in many experimental systems where the use of monoclonal antibodies (mAb) as antagonists has proven inhibitory to multiple events associated with inflammation.

Leukocyte integrins mediate a wide range of adhesion-dependent functions both in antigen-dependent and antigen-independent processes. *In vitro*, mAbs to CD18 or CD11a inhibit lymphocyte mediated lytic events such as cytotoxic T cell activity and natural killer cell activity (Krensky *et al.*, 1983; Sanchez-Madrid *et al.*, 1983; Kohl *et al.*, 1984) and lymphocyte trafficking processes such as attachment of lymphocytes to vascular endothelium and epidermal cells (Haskard *et al.*, 1986; Dustin *et al.*, 1988). Furthermore, these mAbs inhibit antibody formation and mitogen- and antigen-induced T cell proliferation as well as MLR (Davignon *et al.*, 1981; Dougherty *et al.*, 1988; Dougherty and Hogg, 1987; Boyd *et al.*, 1988). All of these processes require cell-cell interactions for successful responses. Moreover, mAbs to CD18 or CD11 also inhibit certain granulocyte functions, such as their attachment to endothelium (Smith *et al.*, 1988), homotypic aggregation (Anderson *et al.*, 1986), binding to iC3b coated particles (Anderson *et al.*, 1986; Beller *et al.*, 1982), and antibody-dependent cellular cytotoxicity (Kohl, 1987).

A second line of evidence suggesting an important role of the leukocyte integrins in inflammatory reactions came from studies of individuals with genetic mutations in the gene

encoding the common β_2 subunit CD18. As a result, their cells are unable to express normal levels of leukocyte adhesion molecules on the cell surface (Anderson and Springer, 1987; Todd and Freyer, 1988; Dustin and Springer 1991; Carlos and Harlan, 1994). Patients with this congenital leukocyte adhesion deficiency type I syndrome (LAD I) have recurring bacterial infections which are fatal in childhood unless corrected by bone marrow transplantation. Neutrophils from these patients fail to migrate in response to chemoattractants and are unable to bind to and cross the endothelium at sites of inflammation. Pus, therefore, fails to form and this seriously compromises the ability of these children to fight infections. The most severely deficient patients (expressing <1% of the normal number of leukocyte integrins) also have depressed T cell responses. This is presumably a result of defective antigen-presenting capacity due to the inability of their T cells to attach effectively to the antigen-presenting cells (APCs). *In vitro*, lymphocytes from these individuals behave just like normal lymphocytes in the presence of anti-LFA-1 mAbs. Their cells also show defective proliferative and effector responses in assays requiring cell-cell interactions. Such observations indicate an even broader role of the leukocyte integrins in antigen-independent inflammatory and immune processes to also include leukocyte migration and diapedesis.

Animal models for LAD I, allowing for experimental manipulation *in vivo*, will be of significant use in studying the *in vivo* function of the leukocyte integrins. A canine LAD I model appearing completely analogous to the human has been identified (Giger *et al.*, 1987). As a second approach, mAbs against the leukocyte integrins have been administered into experimental animals to duplicate a LAD I state. Such studies have shown that peritoneal macrophages are not elicited with thioglycollate in mice that have been injected with anti-Mac-1 mAb (Rosen and Gordon, 1987). Similarly, leukocytes from rabbits injected with anti-CD18 mAb do not bind to endothelium and their extravasation is prevented (Arfors *et al.*, 1987). Both of these studies suggest that leukocyte integrins play an important role in leukocyte extravasation. Furthermore, CD18-mutant mice having 2 or 16% of normal CD18 expression on granulocytes in the resting or activated state, respectively, have been generated (Wilson *et*

al., 1993). These mutant mice show an impaired inflammatory response to a chemical peritonitis and delayed rejection of cardiac transplants (Wilson *et al.*, 1993).

1.1.1 Regulation of ICAM-1 Expression

The LFA-1/ICAM-1 mechanism is regulated through changes in counter-receptor expression in addition to changes in LFA-1 avidity. Regulation of ICAM-1 expression allows control of the spectrum of cells to which activated leukocytes can adhere and in some cases coordinates the ability to adhere with the presence of major histocompatibility complex (MHC) molecules required for foreign antigen presentation (Dustin and Springer, 1991). In most cases, ICAM-1 expression is regulated by cytokine receptors coupled to mechanisms for altering gene expression.

Resting lymphocytes lack significant ICAM-1 expression, but ICAM-1 expression increases during T and B lymphocyte activation (Dustin *et al.*, 1986; Clark *et al.*, 1986; Wawryk *et al.*, 1989). Thus, activated lymphocytes in germinal centers and at sites of inflammation are strongly positive (Dustin *et al.*, 1986; Wantzin *et al.*, 1989). In contrast, monocytes also possess an intracellular store of ICAM-1 which can be mobilized to the cell surface following adherence to fibronectin (Dougherty *et al.*, 1988). Similarly, leukocyte integrins, Mac-1 and p150,95, are stored in intracellular pools in monocytes and polymorphonuclear cells (Todd *et al.*, 1984; Miller *et al.*, 1987; Bainton *et al.*, 1987) and this pool can be rapidly mobilized to the cell surface in response to a variety of chemoattractants including f-Met-Leu-Phe (fMLP), C5a, and leukotriene B4 (Springer *et al.*, 1984; Lanier *et al.*, 1985; Berger *et al.*, 1984; Todd *et al.*, 1984; Miller *et al.*, 1987; Bainton *et al.*, 1987). The intracellular pool of these β_2 integrins has been shown to localize to peroxidase-negative granules in monocytes and granulocytes (Miller *et al.*, 1987; Bainton *et al.*, 1987). However, neither the nature of the compartment in which ICAM-1 is sequestered in monocytes nor the

relationship of this compartment to the peroxidase-negative granules containing rapidly mobilizable Mac-1 and p150,95 is known.

The rapidity of appearance of ICAM-1 that can be induced on nonleukocytes is even more dramatic. ICAM-1 is absent from most cells in normal, nonlymphoid tissues, except for expression of low levels on endothelial cells. Local immune responses result in a rapid increase in ICAM-1 expression on endothelial cells and induction of ICAM-1 on epithelial and mesenchymal cells (Dustin *et al.*, 1986; Dustin and Springer, 1988; Wantzin *et al.*, 1989; Munro *et al.*, 1989). In fact, ICAM-1 expression can be increased on multiple cell types including keratinocytes in inflamed skin lesions (Wantzin *et al.*, 1989; Griffiths and Nickoloff, 1989), transplanted liver bile duct cells and perivenular hepatocytes during rejection (Adams *et al.*, 1989), endothelium in the brain surrounding MS plaques (Sobel *et al.*, 1990), transplanted kidney glomeruli and tubules during rejection (Cosimi *et al.*, 1990), and lung epithelial cells following antigen provacation (Wegner *et al.*, 1990). These *in vivo* results are correlated with the ability of products of activated lymphocytes (lymphokines) and monocytes (monokines) to increase ICAM-1 expression on cultured fibroblasts, endothelial cells, epithelial cells, and astrocytes (Dustin *et al.*, 1986, 1988; Dustin and Springer, 1988; Pober *et al.*, 1986; Frohman *et al.*, 1989). The increased expression of ICAM-1 seen on malignant melanomas and carcinomas may be secondary to local immune reactions generating cytokines (Temponi *et al.*, 1989; Vogetseder *et al.*, 1989; Holzmann *et al.*, 1988; Natali *et al.*, 1990).

1.1.2 Transcriptional Regulation of ICAM-1 Gene Expression

The proinflammatory cytokines, IFN-γ, TNF-α, and IL-1, as well as LPS and the protein kinase C (PKC) activator PMA, can markedly increase the expression of ICAM-1 on various cells of both hematopoietic and non-hematopoietic origins (Dustin *et al.*, 1986; Rothlein *et al.*, 1988). Because IFN-γ enhances the transcription of many IFN-γ-responsive genes including

the class II MHC (Moses *et al.*, 1992), in combination with the fact that ICAM-1 was initially noted to have a tissue distribution similar to that of the MHC class II antigens (Dustin *et al.*, 1986), *i.e.*, vascular endothelium, germinal center cells, interdigitating reticulum cells, macrophages, lymphoid tissues, and epithelial cells in tonsil and thymus, its effect on ICAM-1 was thought to be similar. Indeed, within the 5'-flanking region of ICAM-1 gene are contained potential IFN-γ-responsive elements, as well as glucocorticoid receptor binding sites, NF-κB consensus sequences, and AP1/TPA-responsive element (TRE)-, AP-2- and AP-3-like sites (Degitz *et al.*, 1991; Voraberger *et al.*, 1991). However, in a reporter assay these potential elements could only mount a two-fold induction with IFN-γ stimulation (Voraberger *et al.*, 1991), suggesting that ICAM-1 gene regulation by IFN-γ may also involve posttranscriptional mechanisms. Similarly, PMA was found to have little effect on the transcription of the ICAM-1 gene in human umbilical vein endothelial cells (HUVECs) as measured by a nuclear run-on assay (Wertheimer *et al.*, 1992). In contrast, Voraberger *et al.* (1991) reported that PMA stimulated the expression of a luciferase reporter gene linked to the 5'-flanking region of the ICAM-1 gene containing three copies of TRE in transiently transfected A549 cells. Interestingly, similar results were obtained in HUVECs transfected with the same pBHluc 1.3 construct of Voraberger *et al.* (1991) suggesting that, although PMA can activate exogenous ICAM-1 enhancer/promoter elements in HUVECs, the same elements in the endogenous gene remain functionally silent (Wertheimer *et al.*, 1992).

Other proinflammatory cytokines such as TNF-α and IL-1, however, are thought to induce ICAM-1 message primarily at a transcriptional level (Voraberger *et al.*, 1991; Wertheimer *et al.*, 1992). Activation of the ICAM-1 gene by TNF-α as measured by nuclear run-on studies, is both rapid and transient, with peak transcription rates occurring at 30 min and returning to basal level within 2 h, thus providing the cell with a burst of ICAM-1 messenger RNA (mRNA) synthesis (Wertheimer *et al.*, 1992). Moreover, TNF-α is known to activate the ubiquitously expressed transcription factor NF-κB, which plays a role in the inducible expression of many genes (Lenardo and Baltimore, 1989). As mentioned above,

several potential NF-κB binding sequences have been identified in the 5'-regulatory region of the ICAM-1 gene, and TNF-α has been shown to promote nuclear NF-κB-like binding activity in HUVECs (Montgomery *et al.*, 1991). However, Wertheimer *et al.* (1992) found that NF-κB binding activity is also induced in PMA-stimulated HUVECs without a concomitant increase in ICAM-1 gene transcription, indicating that NF-κB activation alone is insufficient to induce the transcription of ICAM-1 gene. Similarly, TNF-α-stimulated activation of NF-κB is insufficient to activate ELAM-1 gene transcription in HUVECs (Montgomery *et al.*, 1991). Thus, even the mechanisms for the regulatory effects of TNF-α on ICAM-1 expression do not appear to be fully understood and require further characterization of TNF-α-responsive *cis*-acting elements of the ICAM-1 gene.

1.2 ICAM-2

LFA-1-dependent antigen-specific responses can be completely inhibited, unaffected, or partially inhibited by ICAM-1 mAbs depending on the target cell used. This phenomenon predicted the existence of at least one alternative counter-receptor for LFA-1. Such a second ligand, ICAM-2 (CD102), was formally identified by screening for cDNA clones which, when expressed, would promote cell adherence to purified LFA-1 in the presence of mAb to ICAM-1 (Staunton *et al.*, 1989b). ICAM-2 is another member of the Ig superfamily that is expressed on endothelium and is involved in leukocyte adherence. The gene encoding human ICAM-2 is a single-copy gene located on chromosome 17 (Hogg *et al.*, 1991). Molecular cloning of the ICAM-2 gene showed a core protein of 29 kD with six residues for possible N-linked glycosylation which, if fully used, would yield a mature protein of 46 kD (Staunton *et al.*, 1989b). ICAM-2 is an integral membrane protein with two extracellular Ig-like domains, closely related to ICAM-1 (34% homology with the two NH_2-terminal domains of ICAM-1) (Staunton *et al.*, 1989b). The ligand-binding site for LFA-1 is located in these domains of

ICAM-1; hence, ICAM-2 is a second endothelial ligand for this leukocyte integrin (Staunton *et al.*, 1989b). However, the observation that Mac-1 binds to the third Ig-like domain of ICAM-1 suggests that ICAM-2 does not serve as a ligand for this leukocyte integrin. Unlike ICAM-1, the expression of ICAM-2 is not affected by cytokines (Staunton *et al.*, 1989b; de Fougerolles *et al.*, 1991). ICAM-2 also does not bind to the major or minor group of rhinoviruses (Larson and Springer, 1990).

Murine ICAM-2 has been molecularly cloned and shares 60% identity to human ICAM-2 at the protein level (Xu *et al.*, 1992). However, the transmembrane and cytoplasmic regions of murine ICAM-2 are more highly conserved (75% protein identity). In contrast to human ICAM-2, the murine counterpart has five potential sites for N-linked glycosylation.

1.3 ICAM-3

A third LFA-1 counter-receptor, ICAM-3 (CD50), has also been recently identified (Fawcett *et al.*, 1992; Vazeux *et al.*, 1992; de Fougerolles *et al.*, 1993). ICAM-3 contains 15 potential N-linked glycosylation sites and is a heavily glycosylated protein of 116 to 140 kD in a cell type-specific fashion, with an expected core polypeptide of about 56 kD. Molecular cloning of ICAM-3 revealed that it contains five Ig-like domains that are highly similar to the corresponding domains in ICAM-1 and ICAM-2. ICAM-3 shares 48% overall aa identity with ICAM-1 rising to 77% in domain 2, while the first two NH_2-terminal Ig-like domains of ICAM-3 have 31% aa identity to the two Ig-like domains of ICAM-2.

Despite the homologies between the three ICAMs, their patterns of expression suggest specialized roles. The finding that adhesion of resting T lymphocytes to purified LFA-1 occurs primarily via ICAM-3, combined with the fact that ICAM-3 is expressed constitutively at higher levels on monocytes and resting lymphocytes than other LFA-1 ligands, suggest that ICAM-3 might be important in the initiation of immune responses (Fawcett *et al.*, 1992;

Vazeux *et al.*, 1992; de Fougerolles *et al.*, 1993, 1994). ICAM-3 mAbs effectively inhibit ICAM-3 adhesion to purified LFA-1 and in combination with ICAM-1 and ICAM-2 mAbs inhibit the homotypic aggregation of PMA-induced lymphoid cell lines (de Fougerolles *et al.*, 1994). Moreover, purified ICAM-3 was found to support LFA-1-dependent adhesion, and also to be capable of providing a costimulatory signal to resting T lymphocytes (de Fougerolles *et al.*, 1994). ICAM-3 is important in peripheral blood lymphocyte (PBL) proliferation in response to phytohemagglutinin (PHA), and in allogeneic and antigen-specific proliferation (de Fougerolles *et al.*, 1994). Thus, ICAM-3 could potentially be important in lymphocyte activation, as well as in adhesion.

1.4 MECHANISMS OF EUKARYOTIC mRNA DEGRADATION

Changes in degradation rates among mRNAs contribute significantly to the control of gene expression in eukaryotes. In particular, the existence of highly unstable mRNAs allows for rapid and precise reduction or elevations in message levels following changes in transcript synthesis rates or alterations in the transcript turnover rates. The range of mRNA stability in eukaryotic cells can vary over several orders of magnitude (Peltz *et al.*, 1991). Some mRNAs in higher cells are degraded with a half-life of about 20 min, while other mRNAs are degraded with half-lives over 24 h. Similar variations have been found in yeast, with the most labile messages decaying in less than 5 min and the most stable degrading at rates slower than 60 min (Herrick *et al.*, 1990).

Several examples of regulated mRNA stability in eukaryotic cells have been reported, and these indicate that the trans-acting factors of the message decay system can be subject to regulation. For instance, maturation of T lymphocytes is due in part to the stimulated decay of specific mRNAs, and this probably results from a cascade events involving PKC (Takahama and Singer, 1992). The regulation of transferrin receptor mRNA stability in response to iron

levels is an example of how a destabilizing sequence element can be inactivated by a pathway responding to cellular conditions (Klausner *et al.*, 1993). The cell cycle regulation of histone mRNA levels is an example of how the coordinate synthesis of macromolecules can be controlled by the regulation of mRNA decay (Harris *et al.*, 1991). Furthermore, the stabilization of heat shock protein mRNA at high temperatures demonstrates the efficiency of stabilizing mRNA only when it is needed (Petersen and Lindquist, 1988). Moreover, the significance of message degradation in differential gene expression is exemplified by the transformation phenotype associated with mRNA stabilization mutations in the *c-myc* and *c-fos* mRNAs (Schiavi *et al.*, 1992) and the alterations in mRNA-specific destabilizing factors in monocytic tumors (Schuler and Cole, 1988).

1.4.1 Determinants of mRNA Decay

Recent works on mRNA turnover illustrate that there are sequence elements regulating the decay rate throughout the message. The presence of such a diverse array of elements rules out the simple model that mRNA is passively protected from degradation by nucleases simply by the presence of stabilizing sequences at its ends. This raises the questions of whether there is a common pathway for mRNA degradation or whether the various elements act independently, and also whether they represent sites of nuclease sensitivity or whether they represent recognition sites for other proteins that lead eventually to the recruitment of a common ribonuclease.

1.4.2 The 5' Cap Structure

The unique 5'-5' phosphodiester bond of the cap makes it intrinsically resistant to general ribonucleases, and it is this resistance that allowed its original identification. A specific enzyme that removes the cap (decapping enzyme) has been purified from yeast (Stevens, 1988), and it is possible that an activation of this enzyme by mRNA sequences can create an unstable mRNA. Decay of the mRNA would result owing to the existence of a 5'-3' exoribonuclease or an endoribonuclease whose site of action was masked by the cap structure and its associated cap-binding proteins (Rhoads, 1993).

1.4.3 Translational Requirement of mRNA Degradation:

1.4.3.1 5' Untranslated Region

Although the 5' untranslated region (UTR) of mRNA has not been definitively shown to be a destabilizing sequence on any mRNA, it has been well documented to control the translatability of a message, and it is through the negative regulation of translation initiation that this region of mRNA has been shown to inhibit the degradation process.

Initial understanding of the relationship between translation and mRNA turnover seems to indicate that most mRNAs need to be translated to be degraded. In yeast, almost all mRNAs are stabilized by treatment with the translation inhibitor, cycloheximide (Herrick *et al.*, 1990). Concordantly, a mutation resulting in partial loss of function of a transfer RNA (tRNA) nucleotidyl transferase protein, which leads to a decrease in the rates of translation due to limiting functional tRNA, also results in message stabilization (Peltz *et al.*, 1992). However, these experiments do not distinguish between a requirement for translation in mRNA decay and a requirement for a labile degradative protein activity that fails to be synthesized but continue to be degraded upon inhibition of translation.

Placement of a stable 5'UTR stem-loop structure in various yeast mRNAs can inhibit translation up to 96% without changing the transcript levels and hence, by inference, the stability of the mRNA (Cigan *et al.*, 1988). These data are inconsistent with the translation inhibitor studies and suggest that wild-type rates of translation initiation are not needed for inducing mRNA decay, although it is still unclear what would happen if mRNA becomes untranslatable.

In the work by Koeller *et al.* (1991), the translation of *c-fos* mRNA containing the destabilizing AU-rich region was found to be negatively regulated by at least 95% by an inhibitory sequence, called the iron-responsive element (IRE), located within its 5'UTR. Regulation of translation was achieved by altering the iron levels in the tissue culture media, while the general metabolism and the overall translational capacity of the cell remained unchanged. The results from this study suggested that translation is not required for mRNA degradation.

In contrast, Aharon and Schneider (1993) fused a mutated form of the adenovirus tripartite leader that inhibits translation of a synthetic mRNA construct that was destabilized owing to the presence in its 3'UTR of the AU-rich region of the granulocyte/macrophage colony-stimulating factor (GM-CSF) mRNA. The results from their study indicate that inhibition of translation results in message stabilization. Furthermore, relief of translation inhibition and the subsequent appearance of unstable mRNA is achieved by the introduction of a ribosomal entry site downstream of the 5' inhibitory sequence. In support, Savant-Bhonsale and Cleveland (1992) have found that mRNA containing an AU-rich element (ARE) must be translated to be rapidly degraded.

Reasons to why there are differences in the requirement of translation for mRNA decay are unclear. It has been speculated recently that the requirement for translation in decay is actually a requirement for recruiting translation factors and that these factors, themselves, associate with the degradative enzymes. In support of this model are findings that a protein needed for translation initiation is also a ribonuclease in yeast (Sachs and Deardorff, 1992).

1.4.3.2 Nonsense-mediated mRNA Decay

Premature translational termination caused by nonsense mutations has been shown to accelerate mRNA turnover rates (Peltz *et al.*, 1991). Trans-acting factors involved in such nonsense-mediated mRNA decay have been identified in studies of a novel class of yeast non-tRNA nonsense suppressors (Peltz and Jacobson, 1993; Leeds *et al.*, 1991, 1992). Mutations in two genes that were isolated as allosuppressors, UPF1 and UPF3, lead to the selective stabilization of mRNAs containing early nonsense mutations without affecting the decay rates of most other mRNAs (Peltz and Jacobson, 1993; Leeds *et al.*, 1991, 1992). The UPF1 gene has been cloned and sequenced (Leeds *et al.*, 1992; Altamura *et al.*, 1992) and several structural and functional properties have been identified. It is non-essential for viability; encoding a 109 kD protein with both zinc finger, nucleotide (GTP)-binding site and RNA helicase motifs. It is identical to NAM7, a nuclear gene that was isolated as a high copy suppressor of mitochondrial RNA-splicing mutations (Altamura *et al.*, 1992). It is also partially homologous to the yeast SEN1 gene (Leeds *et al.*, 1992; Altamura *et al.*, 1992). The latter encodes a non-catalytic subunit of the tRNA-splicing endonuclease complex (Leeds *et al.*, 1992), suggesting that Upf1p (the UPF1 gene product) may be part of a nuclease complex targeted specifically to nonsense-containing mRNAs.

An explanation for why nonsense mutations exhibit a loss of destabilizing activity as they are moved further away from the initiator methionine has recently been shown. By utilizing either wild-type or mutant UPF1 strains, Peltz *et al.* (1993) were able to show that this distance dependence was created by several distinct RNA sequence elements. First, as expected, the presence of a nonsense mutation was essential for UPF-1-mediated decay. Second, the presence of a downstream sequence was also required, and point mutagenesis of this sequence suggested methionine codons were an essential part of it. Finally, the UPF1 pathway was inactivated by the presence of an as yet ill-defined protection sequence preceding the nonsense mutation.

These data are consistent with the model that the transiting ribosome can be made insensitive to nonsense mutations and that sensitivity only occurs when the ribosome has the ability to reinitiate downstream of the nonsense mutation. Nonsense mutations would therefore lose their destabilizing activity as they move further away from the initiator codon because of the increase in probability of being 3' to a protection sequence and not 5' to another initiator codon.

1.4.4 Open Reading Frame Destabilizing Sequences

Several different experiments have shown that the open reading frame (ORF) region of mRNA contains destabilizing sequences. The N-terminal tetrapeptide encoded by the β-tubulin mRNA provides a target signal for rapid degradation of the message under conditions of tubulin monomer excess (Yen *et al.*, 1988). Experiments utilizing the protooncogene *c-fos* mRNA also showed the existence of destabilizing elements within the ORF of its mRNA (Shyu *et al.*, 1989). The 3'UTR of this mRNA also contains a destabilizing sequence, but Shyu *et al.* (1989) found that removal of this 3'UTR element was not sufficient to stabilize *c-fos* mRNA. Contemporaneously, another destabilizing sequence within the ORF was identified that was utilized only when the message was transiently expressed following growth factor stimulation. A closer examination of this region has shown that it contains two destabilizing elements, and that translation through this region is required for inducing degradation. (Wellington *et al.*, 1993).

Furthermore, a series of proteins that recognize the ORF destabilizing region of *c-fos* have been identified by ultraviolet cross-linking experiments (Chen *et al.*, 1992). In related experiments examining an analogous destabilizing sequence in ORF of *c-myc* mRNA, Bernstein *et al.* (1992) found the *in vitro* decay rates of the polyribosome-bound *c-myc* mRNA to be dramatically increased in the presence of excess synthetic RNA containing the

destabilizing region. This activation presumably results from the competition for a 75 kD protein that recognizes the destabilizing sequence and inactivates it. These *c-fos* and *c-myc* studies provide some evidence for the importance of protein complex formation in the regulation of ORF destabilizing determinants.

1.4.5 3'UTR Destabilizing Elements

The two most well characterized examples of destabilizing sequences within the 3'UTR are the IRE on the transferrin mRNA and the ARE found on many unstable mRNAs. Located within the 3'UTR of the transferrin mRNA is a region that contains five distinct stem-loop structures capable of binding the IRE-binding protein (IRE-BP) (Klausner *et al.*, 1993). The affinity of this protein for these sequences is regulated by cellular iron, and changes in affinity occur through dissociation and reassociation of an iron-sulfur cluster within the IRE-BP. The binding of the IRE-BP to transferrin mRNA stabilizes this mRNA. However, the IRE structure itself does not destabilize the mRNA, but is instead made unstable owing to the presence of an as yet uncharacterized destabilizing sequence in the vicinity of the stem-loop structures. It is, therefore, thought that the binding of IRE-BP to the IREs prevents association of destabilizing factors to a cryptic destabilizing sequence. Whether these destabilizing factors are themselves ribonucleases, or whether they recruit ribonucleases to degrade the mRNA at a distant site, remains unclear.

The regulation of transferrin mRNA decay provides two important mechanistic insights. First, it shows how the decay process can be regulated by proteins that can directly respond to changes in the cellular environment. Second, the transferrin mRNA studies reveal that mRNA stabilization may, in fact, result from an inhibition of an activity recognizing the destabilizing element and that this destabilizing element can be distant from the binding site of the stabilizing proteins.

An example of the inhibition of mRNA destabilizing sequences by a distant sequence element has also been described (Heaton *et al.*, 1992). A 3'UTR destabilizing sequence from the yeast STE3 mRNA was found to stimulate decay of the normally stable yeast PGK1 mRNA only when a portion of the PGK1 ORF had been removed. The inability of a destabilizing sequence to work in certain contexts raises the possibility that the interaction between different regions of mRNA can lead to changes in the efficiency of element utilization. Such regulation in *cis* by DNA sequence elements surrounding transcriptional promoters is well documented. These data thus provide evidence that a number of mRNA metabolism reactions may be subject to similar hierarchical controls.

The second well characterized 3'UTR destabilizing sequence is the ARE. Characteristic classes of ARE-containing cellular mRNAs include the lymphokine and the immediate early genes, including several protooncogenes (Bohjanen *et al.*, 1991, 1992; Brewer, 1991; Caput *et al.*, 1986; Gillis and Malter, 1991; Shaw and Kamen, 1986; Vakalopoulou *et al.*, 1991; Wilson and Triesman, 1988; Shyu *et al.*, 1991; You *et al.*, 1992; Myer *et al.*, 1992; Schuler and Cole, 1988). A number of groups have identified and begun to characterize factors ranging from 20 to 70 kD that bind to these AREs (Bohjanen *et al.*, 1991, 1992; Brewer, 1991; Gillis and Malter, 1991; Vakalopoulou *et al.*, 1991; You *et al.*, 1992; Myer *et al.*, 1992; Schuler and Cole, 1988). Properties of some of these ARE-binding factors suggest a role in mRNA turnover. These include their selective binding to a subset of differentially regulated ARE-containing mRNAs (Bohjanen *et al.*, 1991; Schuler and Cole, 1988), changes in binding activity that correlate with changes in specific mRNA abundance (Bohjanen *et al.*, 1991), and specific degradative activity *in vitro* (Brewer, 1991). Although the AREs of many transcripts share a common AUUUA motif (Shaw and Kamen, 1986), this sequence is not sufficient to confer specificity of binding for all of these factors. For example, Bohjanen *et al.* (1991) have identified a cytoplasmic factor synthesized by stimulated T cells that binds specifically to AUUUA multimers present in the 3'UTR of GM-CSF, IL-2, and TNF-α mRNAs, but does not bind to the ARE in the *c-myc* mRNA. Consistent with the

hypothesis that sequences other than the AUUUA motif can provide specificity , factors have been identified that bind to a 20 nucleotide U-rich region of the *c-fos* 3'UTR that lacks any AUUUA sequences (You *et al.*, 1992). Furthermore, Brewer (1991) has identified an activity that binds to the *c-myc* ARE, or to poly(U), and specifically accelerates the turnover of the *c-myc* message in an *in vitro* mRNA decay system. The 32 kD ARE-binding polypeptide from nuclear extracts identified by Vakalopoulou *et al.* (1991) also requires the AUUUA motif within an U-rich element of several unstable mammalian mRNAs.

From the above experiments, several generalizations can be made. First, it is clear that both nuclear and cytoplasmic proteins can bind specifically to the ARE. The significance of the nuclear-binding proteins remains obscured by a lack of knowledge concerning the contribution of nuclear mRNA processing reactions to the ultimate fate of mRNA in the cytoplasm. Second, the binding activity of some of these proteins appears to increase or decrease in response to changes in cellular metabolism that lead to alterations in the stabilities on ARE-containing mRNAs. Finally, the existence of a 20S complex on an unstable ARE-containing mRNA and the absence of this complex on ARE-containing mRNAs that are stable owing to ARE inactivation (Savant-Bhonsale and Cleveland, 1992) suggest that ARE activity may be mediated by a complex of proteins.

Although the role played by ARE-binding proteins is not yet clear, the mechanism by which this element stimulates decay has been partially defined. Shyu *et al.* (1991) have shown that the ARE in *c-fos* transcripts mediates decay by first stimulating deadenylation and then by providing an element that stimulates the next phase of the decay process. Similarly, several other ARE-containing mRNAs have been found that go through a deadenylation and then a decay process. Thus, deadenylation may be a prerequisite for the decay of this class of unstable mRNAs and the deadenylated mRNAs may represent a key early intermediate in the degradation pathway (Wilson and Triesman, 1988).

1.4.6 Endo- and Exoribonucleases

Identification of the ribonucleases involved will undoubtedly shed additional light on the mechanisms that regulate mRNA degradation. Toward this end, characterization of endo- and exoribonuclease activities that degrade specific mRNAs in cell-free extracts from mammalian (Bandyopadhyay *et al.*, 1990; Wager and Assoian, 1990), chicken (Ling and Jost, 1991), and yeast cells (Vreken *et al.*, 1992), as well as in *Xenopus* oocytes (Brown and Harl, 1990) has begun. In the latter system, specific endonucleolytic cleavage is observed at a repeated sequence within the 3'UTR of a maternal homeobox mRNA (Brown and Harl, 1990). In yeast, two exoribonucleases encoded by the XRN1 and poly(A) nuclease (PAN)1 genes have been characterized recently. The XRN1 gene encodes a 160 kD 5'-3' exoribonuclease that degrades RNAs with 5'monophosphates, but does not digest capped RNAs (Larimer and Stevens, 1990; Stevens and Maupin, 1987; Stevens *et al.*, 1991). Cells that are deleted for XRN1 are viable, but grow slowly, have altered rRNA processing and appear to stabilize the decay of several mRNAs (Larimer and Stevens, 1990; Stevens *et al.*, 1991). It is not clear whether such stabilization is a direct consequence of the deletion of the XRN1 gene on mRNA decay pathways, or a result of reduced rates of growth and protein synthesis.

mRNA polyadenylation occurs in the nucleus of cells, and the posttranscriptionally synthesized tail is typically homogeneous in length, ranging form 70-90 nt in yeast to 220-250 nt in mammalian cells (Reviewed in Sachs, 1990). Following transport of the mRNA, the long poly(A) tail is shortened in the cytoplasm. In yeast, this shortening reaction requires the presence of a specific protein bound to the poly(A) tail, the poly(A)-binding protein (PAB), for full efficiency (Sachs and Davis, 1989), and it is this reaction that could be regulated by the different destabilizing sequences on many RNAs. The poly(A) shortening reaction can be separated into two phases, the first being the shortening of the tail down to 12-25 residues and the second terminal deadenylation step being the removal of some or all of these residues.

The gene encoding the ribonuclease, PAN, involved in PAB-dependent shortening of poly(A) tails from yeast has been cloned (Sachs and Deardorff, 1992). This ribonuclease is unique among identified eukaryotic RNAases in that it requires a protein-RNA complex as a substrate. Furthermore, conditional mutations in PAN reveal that it is also a translation initiation factor. PAN deadenylation activity *in vitro* exhibits the two phases of shortening seen *in vivo*, and both the shortening phase and the terminal deadenylation phase can be regulated by 3'UTR sequences.

Characterization of PAN has revealed some interesting features that may have general implications for RNA degradation. First, the substrate for this ribonuclease is not naked mRNA, suggesting that the search for RNAases that specifically recognize sequences within mRNA that have been identified as destabilizing sequences may be unsuccessful unless the proper ribonucleoprotein substrate is identified. The concept that the nucleolytic substrate is a ribonucleoprotein complex also negates the simple hypothesis that RNA-binding proteins will always protect mRNA from decay. Second, PAN activity can be regulated from distant sites. This argues that destabilizing sequences may not harbor nucleolytic sites, but instead may target sites elsewhere on the mRNA for degradation. A corollary of this is that the identification of endonucleolytic cleavage sites on RNA decay intermediates may not yield information about what region of the mRNA makes these sites nuclease-sensitive. Furthermore, the ability of PAN to be activated or inhibited over distances *in vitro* is consistent with the hypothesis that destabilizing sequences located throughout an mRNA can affect both deadenylation and decay processes. Finally, the requirement for PAN in both translation and presumably RNA decay *in vivo* leads to the suggestion that some of the factors involved in mRNA decay may also be complexed with translation factors. This conclusion is consistent with the apparent requirement for translation in mRNA decay.

1.5 THESIS OBJECTIVES

The regulated expression of ICAM-1 by various tissues and cell types appears to play a vital role in numerous physiologic and pathologic processes of the immune system. Modulation of ICAM-1 expression during inflammation by pharmacologic agents might therefore be an attractive target for medical treatments. However, this requires an understanding of the intracellular regulatory elements and signaling pathways that are involved in the increased expression of ICAM-1 that is induced by proinflammatory cytokines. The overall objective of this thesis study was to test the hypothesis that the regulation of ICAM-1 gene expression by two inflammatory mediators, IFN-γ and PMA, involves a posttranscriptional mechanism and then to define the possible roles of phosphorylation pathways and translation in the process by which cycloheximide induces ICAM-1 message stabilization.

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II

POSTTRANSCRIPTIONAL REGULATION OF ICAM-1 GENE EXPRESSION BY IFN-γ AND PMA

2.1 ABSTRACT

Although ICAM-1 is constitutively expressed at a low level on a subpopulation of hematopoietic cells, vascular endothelium, fibroblasts, and certain epithelial cells, it is dramatically increased at sites of inflammation. IFN-γ and PMA are known to increase the expression of ICAM-1 on many cell types. Because both human and murine ICAM-1 mRNAs contain putative destabilizing AUUUA sequences in their 3'UTRs, the role of mRNA stability in the regulation of ICAM-1 gene expression was examined. The treatment of the murine monocytic cell line P388D1, which constitutively expresses ICAM-1 mRNA at a low level, with IFN-γ or PMA rapidly enhanced the level of ICAM-1 mRNA and dramatically prolonged its half-life. To determine whether the putative destabilizing sequences are responsible for this effect of IFN-γ and PMA, fibroblast L cells were transfected with either the full-length ICAM-1 cDNA or a truncated form (ICAM-1Δ3) lacking the putative destabilizing AUUUA sequences. Although ICAM-1Δ3 mRNA was more stable than the full-length ICAM-1 mRNA, IFN-γ treatment induced the accumulation of both mRNA species and prolongation of their half-lives. The transplantation of the ICAM-1 3'UTR into a stable ICAM-2 mRNA rendered it unstable and was unresponsive to IFN-γ. Therefore, the treatment with IFN-γ stabilizes the otherwise labile ICAM-1 mRNA, but the IFN-γ-responsive sequence may at least in part reside within the protein coding region. PMA also upregulated ICAM-1 gene expression by mRNA stabilization. However, unlike IFN-γ, PMA treatment only increased the level of the full-length, but not the truncated, ICAM-1 mRNA. This demonstrates that the

PMA-responsive element is located within the 3'UTR. Furthermore, the effect of PMA on ICAM-1Δ3 mRNA was recovered by ligating multiple AUUUA sequences derived from a heterologous gene fragment. The stability of this chimeric mRNA and the full-length ICAM-1 mRNA was markedly increased by PMA treatment, indicating that the AUUUA multimers in the 3'UTR are important in the PMA-induced upregulation of ICAM-1 mRNA.

2.2 INTRODUCTION

ICAM-1 is constitutively expressed at a low level on a subpopulation of hematopoietic cells, vascular endothelium, human fibroblasts, and certain epithelial cells (Dustin *et al.*, 1986), and it is dramatically increased at sites of inflammation (Rothlein *et al.*, 1986; Dustin *et al.*, 1986; Pober *et al.*, 1986). The upregulated expression of ICAM-1 on venule endothelium is thought to facilitate the adhesion and subsequent trans-endothelial migration of leukocytes bearing LFA-1 or Mac-1 into inflammatory tissues (Rosenstein *et al.*, 1991; Smith *et al.*, 1989), as well to as permit appropriate interaction of lymphocytes with cells expressing targeted antigens (Springer, 1990; Wawryk *et al.*, 1989; Dougherty *et al.*, 1988). Various pro-inflammatory mediators including IL-1, TNF-α, and IFN-γ have been found to increase ICAM-1 expression on many cell types and are thought to be responsible for the induction of ICAM-1 expression at inflammatory sites *in vivo* (Rothlein *et al.*, 1986; Dustin *et al.*, 1986; Pober *et al.*, 1986; Rothlein *et al.*, 1988).

Despite the importance of ICAM-1 regulation in various immunologic responses, very little is known about the intracellular mechanisms controlling its expression. Potential IFN-γ-responsive elements have been identified within the 5'-flanking region of the ICAM-1 gene (Degitz *et al.*, 1991; Voraberger *et al.*, 1991). However, only a two-fold induction was achieved by IFN-γ using a reporter assay (Voraberger *et al.*, 1991), suggesting that ICAM-1 gene regulation by IFN-γ may involve posttranscriptional mechanisms. PKC has also been

implicated in the induction of ICAM-1, since active phorbol esters such as PMA are known to activate PKC as well as to upregulate cell surface ICAM-1. Furthermore, it has been demonstrated that PMA acts posttranscriptionally to decrease the degradation rate of ICAM-1 mRNA through a PKC-dependent pathway, allowing the constitutively produced message to accumulate (Wertheimer *et al.*, 1992). However, the PMA-responsive element on the ICAM-1 mRNA has not yet been identified.

There is growing evidence that mRNA turnover plays an important role in regulating gene expression. The steady-state levels of many mRNAs do not necessarily reflect their transcriptional rates, suggesting that cellular metabolism is influenced by the stability of individual mRNAs and by the ability of the cells to regulate mRNA turnover. In fact, the rapid turnover of an mRNA ensures that it is maintained at relatively low steady-state levels and that changes in the rate of degradation can affect its steady-state level over a short period of time. This type of regulation allows transient alterations in the expression of some cytokines, transcription factors, and proto-oncogenes such as *c-fos*, *c-myc*, and *c-myb* in response to growth factors, phorbol esters, antigen stimulation, or inflammation (for review, see Sachs, 1993). This class of short-lived mRNAs all share a common AUUUA sequence motif in their 3'UTRs, which serves as one signal targeting the mRNAs for rapid turnover. The 3'UTRs of both human and murine ICAM-1 mRNAs also contain several AUUUA sequences. However, its role in the regulation of ICAM-1 mRNA turnover has not been determined.

The present investigation was intended to define the intracellular mechanisms underlying the inducible expression of ICAM-1 by two inflammatory mediators, IFN-γ and PMA. Our results demonstrate that IFN-γ as well as PMA upregulate ICAM-1 gene expression at least in part at a posttranscriptional level by stabilizing labile ICAM-1 mRNA. Moreover, the stabilization of ICAM-1 mRNA by PMA requires the 3'UTR and may be mediated by the AUUUA destabilizing sequences, whereas the effect of IFN-γ on ICAM-1 mRNA stability appears to involve unknown sequences within the protein coding region.

2.3 MATERIALS AND METHODS

Cell culture. The murine monocytic cell line P388D1 (Koren *et al.*, 1975) was cultured in protein-free hybridoma media II (GIBCO, Grand Island, NY). The mouse fibroblast L cell line was maintained in DMEM containing 10% FCS and antibiotics.

Cloning of murine ICAM-2 cDNA. Total RNA was isolated from EL-4 cell line and reverse transcribed with M-MLV reverse transcriptase using oligo dT primer. Using the following degenerate oligonucleotides, 5'-GTCAACTG(C/T)AG(C/T)(A/T)CC(T/A)C(A/C)T G-3' and 5'-CACAG(T/C)(G/C)(C/A)G(G/A)CAGGAGAA(A/G)TT-3', the cDNA was subjected to 30 rounds of polymerase chain reaction (PCR) amplification (94°C, 30 sec; 50°C, 1 min; 72°C, 1 min) with *Taq* polymerase. The resulting 450 bp DNA fragments were subcloned into pBST and sequenced. Approximately 10% of the clones had homology to the human ICAM-2 cDNA sequence (Staunton *et al.*, 1989). One of these clones was used to screen a λ ZAPII mouse lung cDNA library (Stratagene, La Jolla, CA) with a stringency wash of 0.5x SSC/0.1% SDS at 55°C. G3-1.1 clone with an identical sequence to that of murine ICAM-2 cDNA (Xu *et al.*, 1992) was obtained.

Expression vectors. Expression vectors were constructed using standard recombinant DNA techniques (Sambrook *et al.*, 1989). Briefly, K4-1.1 ICAM-1 cDNA (Horley *et al.*, 1989) was partially digested with *PstI* to remove its 3'UTR, generating the truncated ICAM-1Δ 3 cDNA. The chimeric ICAM-Fas cDNA was constructed by generating the transmembrane and cytoplasmic domains of Fas cDNA (Watanabe-Fukunaga *et al.*, 1992) by PCR using 5'-AAGGGATCCAAAGCTGGGTA-3' and 5'-AGTCCCGGGAAATCGCCTAT-3' oligonucleotides with the *SmaI* and *BamHI* incorporated restriction sites, respectively. The PCR product was then ligated between the *BamHI* and *SmaI* sites of pBST(sk) generating the pBST(sk)-Fas plasmid. The *EcoRI-ScaI* fragment containing the 5 extracellular domains of ICAM-1 cDNA was ligated between the *EcoRI* and *SmaI* sites of pBST(sk)-Fas, creating the ICAM-Fas chimeric cDNA. The ligated junction between the two cDNAs was sequenced to

ensure that it was in frame. The chimeric ICAM-2/1 cDNA was constructed by generating the 3'UTR of ICAM-1 cDNA by PCR using 5'-CCATCGATGCCTGCTGGATGAGA CTCCTGC-3' and 5'-CCATCGATAGACTCTCACAGCATCTGCAGC-3' oligonucleotides. The PCR product was then ligated into the unique *BstXI* site in the 3'UTR of ICAM-2. The *SalI-NotI* isolated ICAM-Fas chimeric, full-length ICAM-1, ICAM-1Δ3, and CD18 cDNAs (Wilson *et al.*, 1989) were blunt-ligated into a stable mammalian expression vector, pRC6 (derived from pAX114; Kay and Humphries, 1991) with a hygromycin resistance gene driven by a thymidine kinase promoter). ICAM-2 and ICAM-2/1 cDNAs were isolated from pUC18/13 with *SalI* and ligated into the *SalI* site in pRC6. *Escherichia coli* MC1061/p3 (Yamasaki *et al.*, 1988) was used for transformation. All the constructs used in this study are schematically shown in Fig. 1.

Transfection and analyses of fibroblast L cells. ICAM-1, ICAM-1Δ3, ICAM-Fas, ICAM-2, ICAM-2/1, and CD18 cDNAs in expression vectors were transfected into ICAM-1, -2, and CD18 negative L cells by the calcium phosphate method (Sambrook *et al.*, 1989). Transfectants were isolated under hygromycin selection (250 μg/ml; Calbiochem, La Jolla, CA). Bulk populations (mixture of individual clones allowed to grow to confluency) as well as clones of transfectants were isolated and characterized. The surface expression of ICAM-1 and -2 was tested by flow cytometry. Briefly, ICAM-1, ICAM-1Δ3, and ICAM-Fas transfected L cells were harvested with PBS containing 2.5 mM EDTA and then stained with YN1/1.7 anti-ICAM-1 hybridoma supernatant (Horley *et al.*, 1989) containing 0.1% sodium azide and goat anti-rat Ig-FITC as a secondary antibody (Cooper Biomedical, West Chester, PA). ICAM-2 and ICAM-2/1 transfected L cells were stained with rat anti-mouse ICAM-2 mAb (PharMingen, San Diego, CA) and goat anti-rat Ig-FITC. The copy numbers of the transfected ICAM-1 and ICAM-1Δ3 genes were determined by genomic Southern blot analysis (Sambrook *et al.*, 1989) using *BamHI* digest and scanning densitometry of the autoradiogram. CD18 transfected L cell clones were detected by a standard Northern analysis (Sambrook *et al.*, 1989).

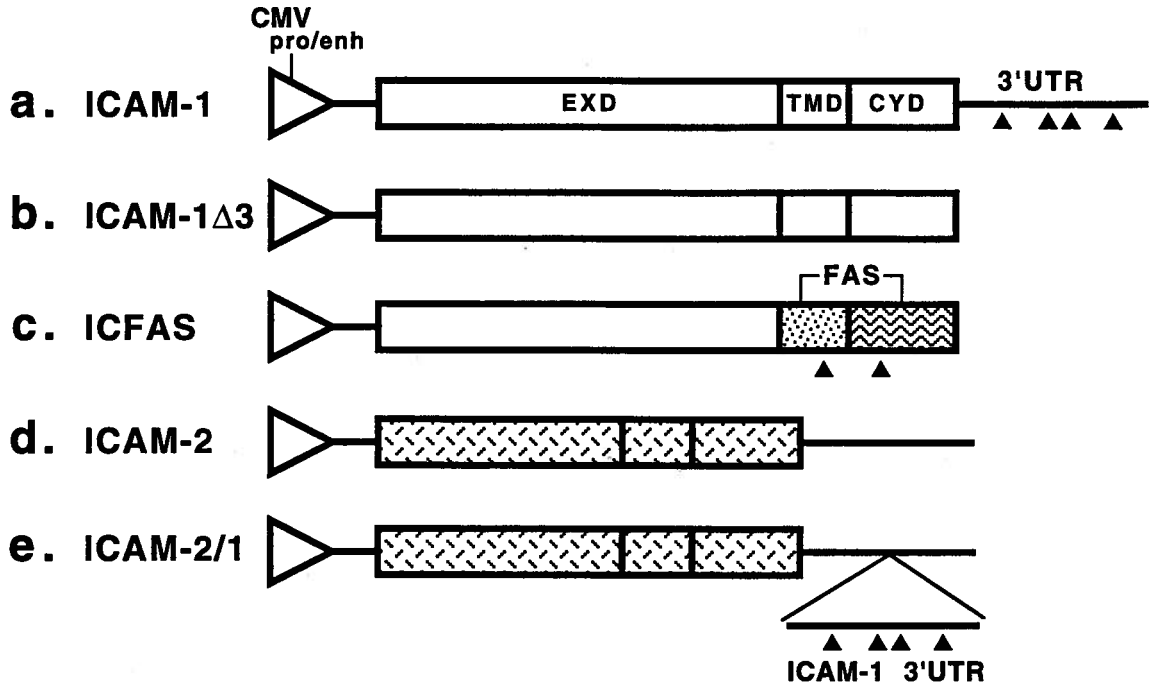


Fig. 1 Expression vectors. **a.** Schematic diagram of the full-length ICAM-1 expression vector. Open triangle represents CMV promoter/enhancer; Solid triangles, AUUUA pentamers; EXD, extracellular domain; TMD, transmembrane domain; CYD, cytoplasmic domain. **b.** Schematic diagram of the truncated ICAM-1Δ3 expression vector. **c.** Schematic diagram of ICAM-1/Fas (ICFAS) chimeric expression vector. Dotted and wavey rectangles represent Fas transmembrane and cytoplasmic domains, respectively. **d.** Schematic diagram of ICAM-2 expression vector. Dashed rectangles represent ICAM-2 cDNA. **e.** Schematic diagram of ICAM-2/1 chimeric expression vector.

Genomic DNA isolation and Southern blot analysis. In order to isolate genomic DNA (Gross-Bellard *et al.*, 1977), cells were washed 3x in PBS. Approximately 5 x 10^7 cells were suspended in 2 ml of TNE buffer (10 mM Tris pH 8.0, 150 mM NaCl, 10 mM EDTA) and gently mixed. To this suspension, 20 µl of 20% SDS and 10 µl proteinase K (stock 10 mg/ml; Sigma, St. Louis, MO) were added and incubated at 37°C overnight. The lysate was then extracted 3x with TNE-saturated-phenol, 3x with TNE-saturated-phenol:chloroform (1:1), and finally 3x with chloroform:isoamyl alcohol (24:1). The aqueous phase was dialyzed against two changes of 4L of TE buffer (10 mM Tris pH 8.0, 1 mM EDTA) each for 24 h at 4°C. The DNA was quantitated spectrophotometrically (A_{260} = 1.0 for 50 µg/ml) and purity was determined ($A_{260}/A_{280} - 2.0$) on an LKB Ultrospec 4050 (Cambridge, England).

Digested genomic DNA (Sambrook *et al.*, 1989) was loaded onto a 0.8% agarose gel and electrophoresed for 16-18 h at 35 volts. Molecular weights were determined from molecular weight standards such as λ *Hind III* fragments or λ *Hind III/EcoRI* fragments. The gel containing digested DNA was transferred onto a piece of Zeta Probe (Bio-Rad Laboratories, Mississauga, ON). DNA was fixed on the membrane by UV cross-linking in a UV Stratalinker 1800 (Stratagene, La Jolla, CA). The blots were prehybridized and then hybridized with 1 × 10^6 cpm/ml of [α^{32}P] ICAM-1 cDNA labeled by random priming (Sambrook *et al.*, 1989). The blots were exposed to Kodak XAR-5 film at -70°C using an intensifying screen.

ICAM-1 induction by IFN-γ, PMA, and cycloheximide. 1.5 × 10^6 P388D1 and the transfected L cells were treated with or without murine IFN-γ (100 u/ml; Boehringer Mannheim, Laval, PQ) or PMA (10 ng/ml; Sigma, St. Louis, MO) or cycloheximide (10 µg/ml; Sigma, St. Louis, MO) and incubated at 37°C for increasing periods of time. The level of mRNA was then determined by Northern blot analysis as described below.

mRNA half-life determination. 1.5 × 10^6 P388D1 and the transfected L cells were treated with actinomycin D (10 µg/ml; GIBCO, Grand Island, NY) for increasing periods of time, and the level of mRNA was determined by Northern blot analysis as described below.

RNA preparation and Northern blot analysis. Total RNA was prepared as described by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987) with minor modifications. Briefly, 1.5×10^6 cells were washed twice with PBS and the pellet was dissolved in 0.5 ml of Solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol). 50 µl of 2 M sodium acetate pH 4.0, 0.5 ml water-saturated-phenol, and 0.1 ml chloroform : isoamyl alcohol (49:1) were then added to the solution and spun at 1.4×10^4 g for 15 min at 4°C. The aqueous layer was mixed with 0.5 ml isopropanol and put at -20°C for 1 h. The solution was then centrifuged for 20 min at 4°C and the pellet was suspended in 300 µl Solution D followed by 300 µl isopropanol. After 1 h at -20°C, the solution was centrifuged for 20 min at 4°C. The pellet was washed with 75% ethanol and then dissolved in 10 µl distilled water.

Equalized aliquots of total RNA (approximately 10 µg) were subjected to electrophoresis in 1% formaldehyde-agarose gels (Sambrook *et al.*, 1989) and transferred onto nylon membranes (Bio-Rad Laboratories, Mississauga, ON). RNA was fixed on the membrane by UV cross-linking in a UV Stratalinker 1800 (Stratagene, La Jolla, CA). The blots were prehybridized and then hybridized with 1×10^6 cpm/ml of [α^{32}P] ICAM-1 cDNA labeled by random priming (Sambrook *et al.*, 1989). The blots were exposed to Kodak XAR-5 film at -70°C using an intensifying screen.

For the induction analysis, the bands on the autoradiogram were integrated with a densitometer by using suitable exposures, and then the values were normalized to the signal of the control β-actin probe or to the constitutively expressed rRNA gene. The maximum intensity was then arbitrarily set at 100% mRNA level as a point of reference. For the mRNA half-life analysis, the integrated band values were normalized to the constitutively expressed rRNA gene, and then the 100% mRNA level was set at time 0.

2.3 RESULTS

Effects of IFN-γ and PMA on ICAM-1 mRNA stability. The treatment of the murine monocytic cell line P388D1 with IFN-γ or PMA induced a rapid increase in the level of ICAM-1 mRNA (Fig. 2a). The maximum increase (5-8x basal level) was achieved within 2 h after the addition of IFN-γ and around 6 h after the addition of PMA. Because both human and murine ICAM-1 mRNAs contain multiple AUUUA destabilizing sequences, we examined the effects of IFN-γ and PMA on the half-life of ICAM-1 mRNA. P388D1 cells were treated with the transcription inhibitor actinomycin D and the decrease in the level of ICAM-1 mRNA was analyzed. Northern blot analysis showed that IFN-γ and PMA treatments clearly prolonged the half-life of ICAM-1 mRNA, extending it from approximately 50 min to far greater than 2 h (Fig. 2b), suggesting that the upregulation of ICAM-1 gene expression by IFN-γ and PMA was due at least in part to the stabilization of ICAM-1 mRNA. As reported for many other labile mRNAs (Sachs, 1993), treatment with cycloheximide, a potent inhibitor of translation, induced a rapid accumulation of ICAM-1 mRNA (Fig. 2c).

Role of AUUUA multimers on induction of ICAM-1 mRNA. To further investigate the role of the AUUUA repeats in the upregulation of ICAM-1 mRNA levels, the murine ICAM-1 cDNA clone K4-1.1 was modified to remove its 3'UTR, subcloned into the mammalian expression vector pRC6 (see Materials and Methods; Fig. 1) and then transfected into murine fibroblast L cells, which do not express detectable levels of ICAM-1 mRNA regardless of IFN-γ treatment. The expression of the transfected cDNA was driven by the CMV promoter and enhancer, which were not responsive to IFN-γ. As a control, the non-inducible murine CD18 cDNA was also transfected into L cells using the same expression vector. Bulk populations of transfected L cells were established by hygromycin resistance. In addition, three randomly picked clones from each type of transfectant expressing the intact or truncated (ICAM-1Δ3) cDNA were isolated and the expression of ICAM-1 on the cell surface was confirmed by flow cytometry. Northern blot analysis of the bulk transfected L cells showed that the basal level of

a

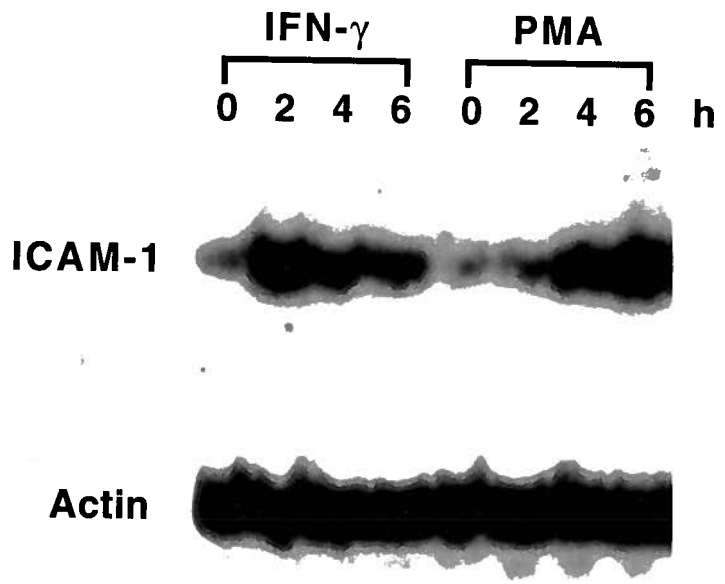
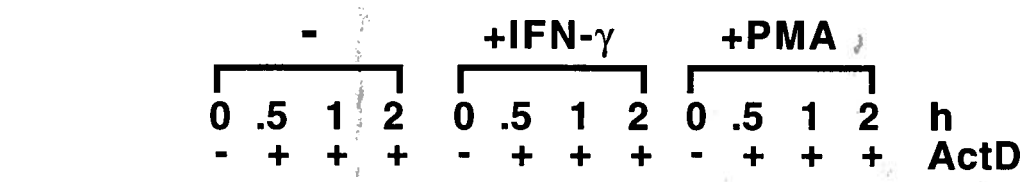


Fig. 2 Effects of IFN-γ and PMA on ICAM-1 mRNA expression in P388D1 cells. Detected by Northern blot analysis. **a.** The induction of ICAM-1 mRNA level in P388D1 cells that had been stimulated with IFN-γ and PMA for the indicated times. The bottom panel represents the corresponding signals of the control β-actin. **b.** Half-life analysis of ICAM-1 mRNA. Cells were treated with IFN-γ for 1 h, PMA for 2 h, or no treatment (–) and then actinomycin D (ActD) was added for the indicated times. **c.** The induction of ICAM-1 mRNA level in P388D1 cells that had been treated with (+) or without (–) cycloheximide (CHX) for 6 h. The bottom panel represents the corresponding signals of the control β-actin. The data presented are representative of two independent experiments.

b



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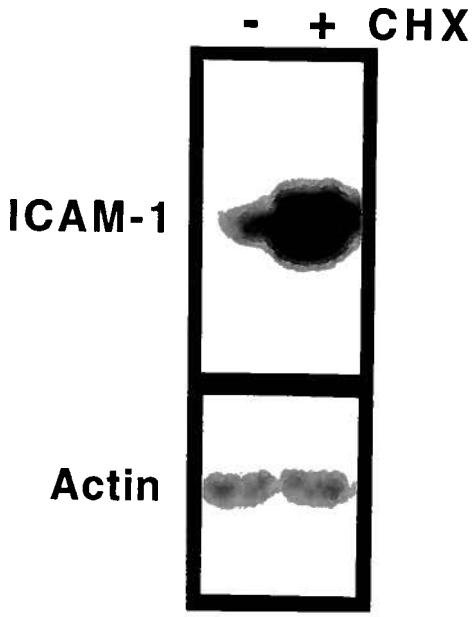


ICAM-1Δ3 mRNA was consistently higher (approximately 4-fold) than that of the intact ICAM-1 mRNA (Fig. 3). Concordantly, the three clones from each type of transfectant gave similar results. The copy numbers of the transfected genes, which may influence the mRNA expression level, in those two groups of transfected L cells, whether bulk or individual clones, determined by genomic Southern blot analysis, were similar to each other (Fig. 4). Therefore, the 3'UTR seemed to regulate the basal level of ICAM-1 mRNA in the transfected cells.

We also examined whether IFN-γ treatment upregulates the level of ICAM-1 mRNA in the transfected L cells. Northern blot analysis showed that the level of ICAM-1 mRNA was elevated by IFN-γ treatment in both types of bulk transfected L cells (Fig. 3). The degree of upregulation of ICAM-1 mRNA levels appeared to be higher in the intact cDNA-transfected L cells (6-9x basal level) than that in L cells with ICAM-1Δ3 cDNA (approximately 3x basal level). This difference however was primarily due to different basal levels between the two ICAM-1 mRNA species, as stated above. L cells transfected with CD18 cDNA using the same vector showed no change in the level of CD18 mRNA upon treatment with IFN-γ, indicating that it has no effect on the transcription of the transfected genes driven by the CMV promoter/enhancer. The β-actin mRNA content was also unaffected by IFN-γ, further demonstrating the specificity of IFN-γ. These observations were confirmed using three randomly picked clones from each type of transfectant.

Effect of IFN-γ on ICAM-1 and ICAM-1Δ3 mRNA stability. To determine whether the observed upregulation of ICAM-1 mRNA was due to its enhanced stability, the effect of IFN-γ on the half-life of ICAM-1 mRNA was examined. Without IFN-γ treatment, full-length ICAM-1 mRNA from the bulk transfectants had a short half-life of approximately 50 min (Fig. 5), whereas, the half-life of ICAM-1Δ3 mRNA was significantly longer (approximately 160 min). The treatment of the bulk transfected L cells with IFN-γ dramatically extended the stability of both full-length and truncated mRNAs (Fig. 5). Concordantly, the mean half-lives of full-length ICAM-1 and ICAM-1Δ3 mRNAs, in the absence of IFN-γ, determined from three independent clones of each transfectant were 42 ± 10 min and 109 ± 17 min,

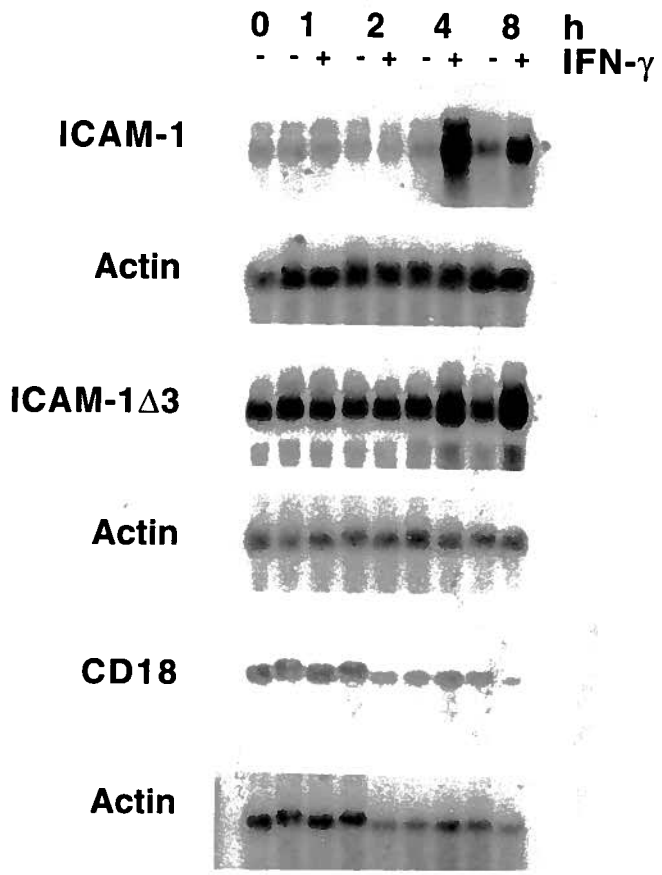


Fig. 3 Effects of IFN-γ on induction of ICAM-1, ICAM-1Δ3, and CD18 mRNA levels. Detected by Northern blot analysis. The induction of ICAM-1, ICAM-1Δ3, and CD18 mRNA levels in the transfected L cells stimulated with (+) or without (–) IFN-γ for the indicated times. The second, fourth, and sixth panels from the top represent the corresponding signals of the control β-actin. The data presented are representative of two independent experiments performed on bulk transfectants. The three independent clones from each type of transfectant gave similar results.

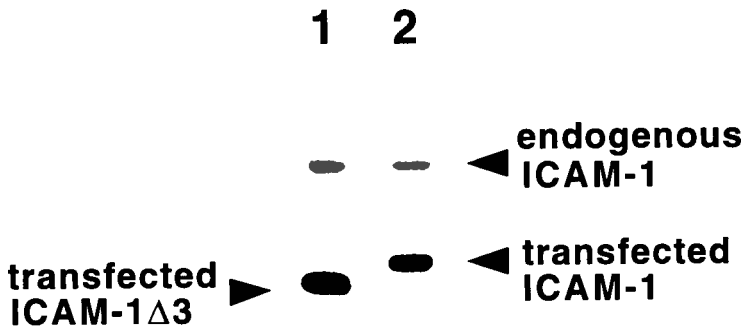


Fig. 4 Southern blot analysis of the exogenous ICAM-1 copy number. Representative *BamHI* genomic Southern blot analysis of ICAM-1Δ3 (lane 1) and ICAM-1 (lane 2) bulk transfected L cells. The genomic DNA from the transfected L cells was digested with *BamHI*, which cuts the vector immediately upstream and downstream of the inserted ICAM-1 and ICAM-1Δ3 gene, and probed with the entire ICAM-1 cDNA. The bulk transfected L cells and the three independent clones of each construct gave bands of similar intensity.

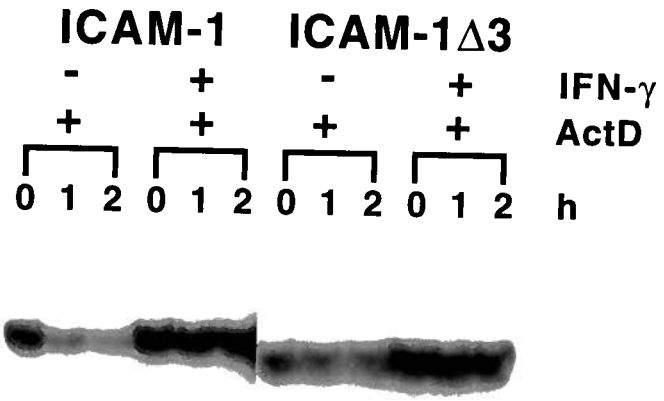


Fig. 5 Half-life analysis of ICAM-1 and ICAM-1Δ3 mRNAs. Detected by Northern blot analysis. The ICAM-1 and ICAM-1Δ3 transfected L cells were either treated with (+) or without (–) IFN-γ for 2 h and then actinomycin D (ActD) was added for the indicated times. The data presented are representative of two independent experiments performed on bulk transfectants. The three independent clones from each type of transfectant gave similar results.

respectively. Hence, ICAM-1Δ3 mRNA was indeed more stable than the full-length ICAM-1 mRNA. Moreover, like with the bulk transfectants, IFN-γ treatment further extended the turnover rates of both transcripts to far greater than 2 h. These results indicate that IFN-γ treatment enhances the level of ICAM-1 mRNA by making it more stable, and this effect occurs even in the absence of the conserved AUUUA pentanucleotide sequences in the 3'UTR, suggesting the presence of a novel IFN-γ-responsive element within the protein coding sequence. This effect of IFN-γ has not been recognized previously.

3'UTR of ICAM-1 mRNA and IFN-γ effects. ICAM-2 is another ligand for LFA-1 but unlike ICAM-1, its expression is constitutive regardless of pro-inflammatory cytokine treatment (Staunton *et al.*, 1989; de Fougerolles *et al.*, 1991). Interestingly, ICAM-2 does not have any AUUUA sequences in its 3'UTR. The murine ICAM-2 cDNA clone G3-1.1 was isolated and a chimeric ICAM-2/1 cDNA (ICAM-1 3'UTR incorporated into ICAM-2 3'UTR) was generated (see Materials and Methods; Fig. 1) to further discern the possible involvement of ICAM-1 3'UTR in the induction and stabilization of ICAM-1 mRNA by IFN-γ. Northern blot analysis of L cells transfected with ICAM-2 or ICAM-2/1 cDNA showed that IFN-γ did not induce the upregulation of these mRNAs (Fig. 6a). Furthermore, the half-life analysis indicated that the ICAM-2 mRNA was rather stable whereas ICAM-2/1 mRNA was relatively unstable (Fig. 6b). Also, IFN-γ had no effect on the stability of either mRNA (Fig. 6b). Thus, these results support the hypothesis that the 3'UTR of ICAM-1 contains mRNA destabilizing elements but by itself is not responsible for the effects of IFN-γ.

Role of AUUUA sequences in PMA-induced accumulation and stabilization of ICAM-1 mRNA. The above results demonstrated that PMA treatment of P388D1 cells upregulates their ICAM-1 expression by a posttranscriptional mechanism that enhances the stability of ICAM-1 mRNA. To determine whether the ICAM-1 3'UTR containing multiple AUUUA sequences is involved in these effects of PMA, we examined the effect of PMA treatment on the accumulation of ICAM-1 and ICAM-1Δ3 mRNA. Northern blot analysis of both bulk and clonal transfectants showed that PMA upregulated the level of full-length ICAM-1 mRNA (5-

a

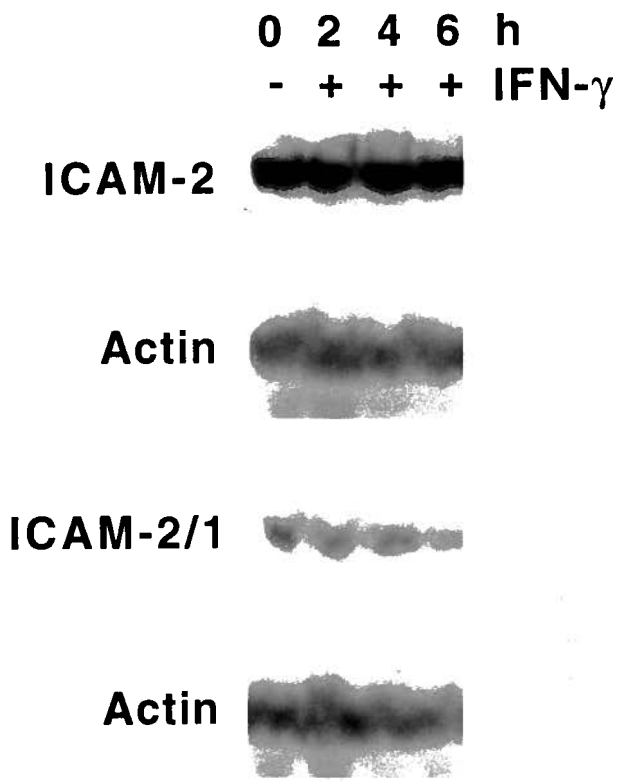
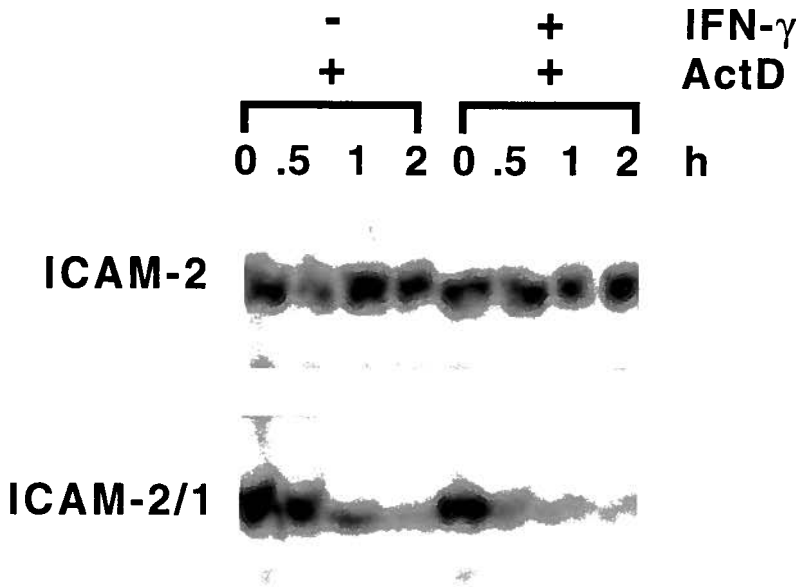


Fig. 6 Effects of IFN-γ on ICAM-2 and ICAM-2/1 mRNA accumulation and stabilization. **a.** ICAM-2 and ICAM-2/1 transfected L cells were treated with IFN-γ for the indicated times. The second and fourth panels from the top represent the corresponding signals of the control β-actin. **b.** Both types of transfected L cells were treated with (–) or without (+) IFN-γ for 2 h and then actinomycin D (ActD) was added for the indicated times. The data presented are representative of two independent experiments.

b



7x basal level) whereas the level of ICAM-1Δ3 mRNA was not affected (Fig. 7). Therefore, the 3'UTR of ICAM-1 containing multiple AUUUA sequences was necessary for the PMA-induced upregulation of ICAM-1 mRNA level.

To further investigate the role of the AUUUA sequences in the the upregulation of ICAM-1 mRNA, ICAM-Fas (ICFAS) chimeric cDNA was constructed, in which the regions of ICAM-1Δ3 cDNA encoding the transmembrane and cytoplasmic domains were replaced by those of Fas cDNA (see Materials and Methods; Fig. 1). As the region of Fas cDNA encoding its transmembrane and cytoplasmic domains contains multiple AUUUA sequences, the resultant ICFAS chimeric cDNA is essentially ICAM-1Δ3 cDNA with AUUUA sequences derived from a heterologous gene fragment. The PMA treatment of L cells transfected with the chimeric cDNA increased the level of ICFAS mRNA (6-fold maximum induction) whereas IFN-γ had no effect (Fig. 8). The half-life analysis showed that PMA stabilized the full-length ICAM-1 mRNA from approximately 50 min to far greater than 2 h (Fig. 9), suggesting that the accumulation of ICAM-1 mRNA by PMA was due to its stabilization. Moreover, the ICFAS mRNA had a half-life of approximately 60 min, which is considerably shorter than the ICAM-1Δ3 mRNA, and PMA treatment greatly enhanced its stability (Fig. 9). These results further suggest that AUUUA sequences may play a role in the general stability of ICAM-1 mRNA, and are sufficient for the upregulation and stabilization of ICAM-1 mRNA induced by PMA whereas the effect of IFN-γ requires the presence of other undefined sequences possibly in the regions encoding the transmembrane and cytoplasmic domains of ICAM-1.

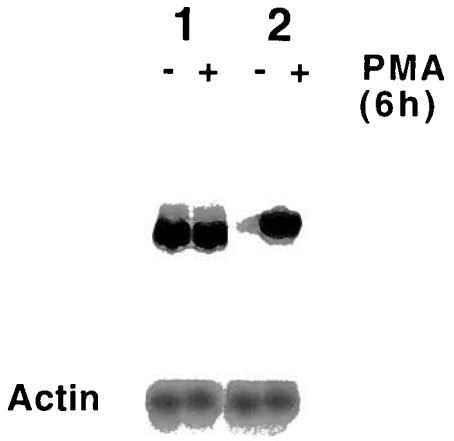


Fig. 7 Effects of PMA on induction of ICAM-1 and ICAM-1Δ3 mRNA levels. Detected by Northern blot analysis. The ICAM-1Δ3 (1) and ICAM-1 (2) transfected L cells were either treated with (+) or without (–) PMA for 6 h. The bottom panel represents the corresponding signals of the control β-actin. The data presented are representative of two independent experiments performed on bulk transfectants. The three independent clones from each type of transfectant gave similar results.

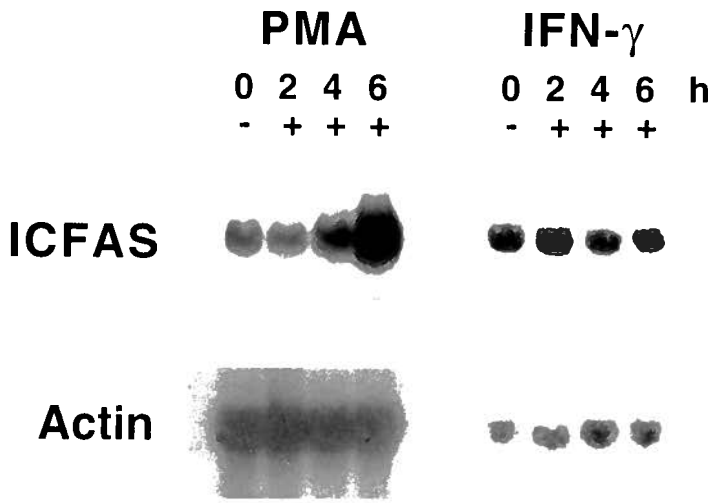


Fig. 8 Effects of PMA and IFN-γ on induction of ICFAS chimeric mRNA level. Detected by Northern blot analysis. The ICFAS transfected L cells were treated with either PMA (left panel) or IFN-γ (right panel) for the indicated times. The bottom panels represent the corresponding signals of the control β-actin. The data presented are representative of two independent experiments performed on bulk transfectants. The three independent clones gave similar results.

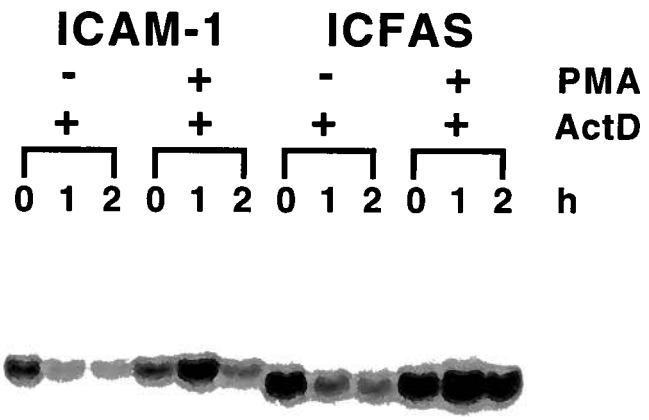


Fig. 9 Half-life analysis of ICAM-1 and ICFAS mRNAs. Detected by Northern blot analysis. The ICAM-1 and ICFAS transfected L cells were either treated with (+) or without (–) PMA for 2 h and then actinomycin D (ActD) was added for the indicated times. The data presented are representative of two independent experiments performed on bulk transfectants. The three independent clones from each type of transfectant gave similar results.

2.5 DISCUSSION

The proinflammatory cytokine IFN-γ as well as the protein kinase C activator PMA dramatically increase the expression of ICAM-1 on various cells (Rothlein *et al.*, 1986; Dustin *et al.*, 1986; Pober *et al.*, 1986; Rothlein *et al.*, 1988). We have shown here that both IFN-γ and PMA upregulate ICAM-1 gene expression at least in part by a posttranscriptional mechanism which stabilizes ICAM-1 mRNA. As expected from the presence of multiple AUUUA destabilizing sequences in its 3'UTR, ICAM-1 mRNA is rather unstable, and the treatment of cells with IFN-γ or PMA clearly stabilizes the mRNA. Interestingly, this effect of IFN-γ can be observed in the absence of the 3'UTR containing AUUUA destabilizing sequences although the effect is less pronounced without the 3'UTR. In support, the incorporation of ICAM-1 3'UTR containing multiple AUUUA sequences into ICAM-2 mRNA converts a previously stable mRNA into a labile transcript. Furthermore, the stability of ICAM-2/1 mRNA remains unaffected by IFN-γ treatment, and hence is unable to induce the accumulation of ICAM-2/1 mRNA. In contrast, the PMA effect on ICAM-1 mRNA stability seems totally dependent on the presence of the AUUUA destabilizing sequences as the presence of exogenous AUUUA sequences derived from the Fas gene appears to be sufficient to mimic the effect of PMA seen on ICAM-1 mRNA stability. These results suggest that at least two pathways regulate ICAM-1 mRNA stability. One is induced by PMA, which is known to activate protein kinase C, and requires the presence of AUUUA sequences. The other, induced by IFN-γ, involves an unknown sequence(s) within the protein coding region. It should be noted that the treatment of cells with cycloheximide also stabilizes and superinduces ICAM-1 mRNA as is the case for many other labile mRNAs (discussed in chapter IV; Ohh and Takei, 1995).

IFN-γ has been known to upregulate ICAM-1 expression on various cells and thought to play an important role in the enhanced expression of ICAM-1 at inflammatory tissues. Because IFN-γ enhances the transcription of many IFN-γ-responsive genes including class II

major histocompatibility complex (Moses *et al.*, 1992), its effect on ICAM-1 has also been thought to be due to its effect on the transcription of ICAM-1 gene. Indeed, putative IFN-γ-reponsive element(s) have been identified in the 5'-upstream region of the ICAM-1 gene (Degitz *et al.*, 1991). However, our current studies have shown that IFN-γ has an addtional role in the upregulation of ICAM-1 gene expression by a posttranscriptional mechanism. This effect of IFN-γ on ICAM-1 mRNA stability has not been recognized previously. On the other hand, PMA treatment has been shown to stabilize the mRNA for ICAM-1 (Wertheimer *et al.*, 1992) as well as the mRNAs for transforming growth factor-β1, IL-1, IL-3, and *c-fms* (Wager and Assoian, 1990; Yomoto *et al.*, 1989; Wodnar-Filipowicz and Moroni, 1990; Weber *et al.*, 1989). Hence, message stabilization by PMA is not unique to ICAM-1. PMA treatment of mononuclear cells resulted in an increased stability of labile messages, such as *c-fos* mRNA which was associated with enhanced adenosine-uridine binding factor (a factor that specifically binds to the AUUUA sequence) (Gillis and Malter, 1991). Conversely, however, stabilization of lymphokine (IL-2, TNF-α, and GM-CSF) mRNAs induced by costimulation with PMA correlated inversely with the binding of a sequence-specific cytoplasmic factor that binds specifically to AUUUA multimers present in the 3'UTR of lymphokine mRNAs (Bohjanen *et al.*, 1991). Here we show that the AUUUA motif in the 3'UTR of ICAM-1 mRNA plays a role in mediating PMA-regulated ICAM-1 mRNA turnover.

Although the relative contribution of mRNA stability, as compared to transcriptional regulation, to the overall ICAM-1 gene expression is not known at this time, it may, nevertheless, be an important regulatory mechanism involved in inflammatory responses. It has been reported that TNF-α upregulates ICAM-1 gene expression at a transcriptional level (Voraberger *et al.*, 1991; Wertheimer *et al.*, 1992) and that IFN-γ upregulates the receptor for TNF-α (Aggarwal *et al.*, 1985; Tsujimoto *et al.*, 1986). This may contribute to the synergistic action of TNF-α and IFN-γ on ICAM-1 mRNA induction (Barker *et al.*, 1990). However, in addition to this process, the present studies suggest that the marked synergism that occurs between TNF-α and IFN-γ may be due to the increased transcription of ICAM-1 gene by TNF-

α and subsequent stabilization of the newly transcribed mRNA by IFN-γ. The regulation of ICAM-1 by mRNA stability could provide a rapid and efficient way of controlling ICAM-1 expression. The rapid turnover of ICAM-1 mRNA ensures that it is maintained at low steady-state levels and that changes in the mRNA stability in response to external stimuli can affect its steady-state level over a relatively short period of time. Such regulation of ICAM-1 may be important for its function. In the absence of an inflammatory response, ICAM-1 is expressed only at a low level on vascular endothelium (Dustin *et al.*, 1986). The upregulated ICAM-1 facilitates the adhesion of leukocytes bearing the receptors for ICAM-1 (namely, LFA-1 and Mac-1) and their subsequent diapedesis into inflammatory tissues (Wawryk *et al.*, 1989; Smith *et al.*, 1989). In comparison, another LFA-1 ligand, ICAM-2, is constitutively expressed on vascular endothelium (Staunton *et al.*, 1989; Xu *et al.*, 1992; de Fougerolles *et al.*, 1991). The basal level of ICAM-2 expression on endothelial cells is much higher than that of ICAM-1, and is not further induced by pro-inflammatory cytokines (Staunton *et al.*, 1989; de Fougerolles *et al.*, 1991). Similarly, the recently cloned ICAM-3 appears to be the primary ligand for LFA-1 on resting lymphocytes, and like ICAM-2, it is expressed in a constitutive manner (Fawcett *et al.*, 1992; Vazeux *et al.*, 1992). Interestingly, both ICAM-2 and -3 mRNAs do not possess AUUUA destabilizing sequences in their 3'UTRs, unlike ICAM-1.

Relatively little is known about the posttranscriptional control of mRNA turnover. The mRNA of the proto-oncogenes *c-myc, c-myb,* and *c-fos* and several cytokines including TNF-α and IFN-γ are very unstable. The recurrent motif in these unstable mRNA involved in their rapid degradation is the pentanucleotide sequence AUUUA that is found singly or in multiple reiteration in the 3'UTR (Sachs, 1993). Proteins (adenosine-uridine binding factor) that bind to these AUUUA sequences have been identified and it has been suggested that these protein-AUUUA complexes may target susceptible mRNAs for rapid cytoplasmic degradation (Sachs, 1993). ICAM-1 mRNA also has the AUUUA repeats in its 3'UTR which seem to influence its stability. However, the stabilization of ICAM-1 mRNA by IFN-γ also occurs in the absence of the conserved sequences in the 3'UTR, implicating a novel mechanism that regulates the

stability of mRNA. Further characterization of the complex regulatory mechanism involved in ICAM-1 expression should enhance our understanding of the inflammatory process and may provide new strategies for modulating the course of inflammatory diseases.

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III

IDENTIFICATION OF IFN-γ- AND PMA-RESPONSIVE ELEMENTS INVOLVED IN ICAM-1 mRNA STABILIZATION

3.1 ABSTRACT

Treatment of cells with IFN-γ or PMA induces upregulation of the level of ICAM-1 mRNA by stabilization of an otherwise labile mRNA. Here, various deletion mutants of ICAM-1 were generated and stably transfected into the murine fibroblast Ltk$^-$ cells that express no endogenous ICAM-1 or -2, in an effort to define the regions within ICAM-1 mRNA that are responsive to IFN-γ or PMA. Induction of ICAM-1 mRNA in the transfected L cells by treatment with IFN-γ revealed that the truncation of the region of ICAM-1 mRNA encoding the cytoplasmic domain made it non-responsive to IFN-γ whereas all other regions were dispensable. In contrast, PMA-induced accumulation of ICAM-1 mRNA required the 3'UTR. To further elucidate the role of these regions in mRNA destabilization and responsiveness to IFN-γ and PMA, ICAM-2 mRNA that is stable and not responsive to IFN-γ or PMA was used as a reporter gene. The putative IFN-γ-responsive region of ICAM-1 mRNA encoding its cytoplasmic domain rendered it unstable and responsive to IFN-γ but not PMA. Conversely, the 3'UTR of ICAM-1 fused with ICAM-2 mRNA also made it unstable and responsive to PMA but not IFN-γ. Half-life analysis showed that the induction of these chimeric mRNAs by IFN-γ and PMA was due, at least in part, to the prolongation of their turnover rate. These results taken together demonstrate that two distinct regions of ICAM-1 mRNA regulate its stability, one encoding the cytoplasmic domain and responsive to IFN-γ, and the other in the 3'UTR and responsive to PMA.

3.2 INTRODUCTION

ICAM-1 is a member of the Ig supergene family and mediates cell adhesion by binding to the leukocyte integrins LFA-1 (Rothlein *et al.*, 1986; Marlin and Springer, 1987; Makgoba *et al.*, 1989) and Mac-1 (Diamond *et al.*, 1991). ICAM-1 is expressed at very low levels on many cell types but its expression is greatly enhanced by various inflammatory cytokines (Dustin *et al.*, 1986; Pober *et al.*, 1986), including IFN-γ, TNF-α, and IL-1, as well as by phorbol esters (Dustin *et al.*, 1986; Rothlein *et al.*, 1988). The enhanced ICAM-1 expression on venule endothelium is thought to enable the adhesion and trans-endothelial migration of leukocytes out of the bloodstream and into the sites of inflammation (Wawryk *et al.*, 1989; Smith *et al.*, 1989). Anti-ICAM-1 antibodies have been demonstrated to inhibit inflammatory responses in a variety of animal models (Barton *et al.*, 1989; Isobe *et al.*, 1992; Cosimi *et al.*, 1990; Wegner *et al.*, 1990). Furthermore, ICAM-1-deficient mice show reduced inflammatory responses (Sligh *et al.*, 1993). Therefore, upregulation of ICAM-1 expression by inflammatory mediators is thought to be an important step in inflammatory responses. However, the intracellular regulatory mechanisms responsible for the inducible expression of ICAM-1 have not been clearly elucidated.

We have previously reported that IFN-γ and PMA stabilize otherwise labile ICAM-1 mRNA and upregulate its expression (Ohh *et al.*, 1994; chapter II). This adds to the growing evidence that mRNA turnover plays an important role in regulating gene expression. This type of regulation allows transient alterations in the expression of some cytokines, transcription factors, and proto-oncogenes such as *c-fos*, *c-myc*, and *c-myb* in response to growth factors, phorbol esters, antigen stimulation, or inflammation (Sachs, 1993; Jackson, 1993). This class of short-lived mRNAs all share a common AUUUA sequence motif in their 3'UTRs, which serves as one signal targeting the mRNAs for rapid turnover (Sachs, 1993; Jackson, 1993). The 3'UTRs of both human and murine ICAM-1 mRNAs also contain several AUUUA sequences and seem to play a destabilizing role (Ohh *et al.*, 1994).

The present study was intended to further define the regions of ICAM-1 mRNA responsive to IFN-γ or PMA. Our results demonstrate that the IFN-γ responsive element resides within the region of 87 nt encoding the cytoplasmic domain of ICAM-1. In contrast, PMA-induced accumulation and stabilization of ICAM-1 mRNA is dependent on the 3'UTR.

3.3 MATERIALS AND METHODS

Cell culture. The murine fibroblast Ltk$^-$ cell line was maintained in DMEM (GIBCO, Grand Island, NY) containing 10% FCS and antibiotics.

Expression vectors. Expression vectors were constructed using standard recombinant DNA techniques (Sambrook *et al.*, 1989). Briefly, K4 ICAM-1 cDNA (Horley *et al.*, 1989) was partially digested with *PstI* to remove its 3'UTR, generating the truncated K4Δ3 cDNA. K4Δc3 cDNA was generated by first digesting the K4 cDNA with *HindIII*, giving the leader, domains 1 to 3, and most of domain 4 (ending at bp 1139). The rest of domain 4 to the end of the transmembrane domain was generated by polymerase chain reaction (PCR) amplification using 5'-TTCAGCTCCGGTCCTGACCC-3' and 5'-TCTAGATCTGGCGGTTATAAACAT-3' oligonucleotides. The two cDNA fragments were ligated together at the *HindIII* site. Generation of sK4 (soluble ICAM-1) cDNA has been described (Welder *et al.*, 1993). K4ld3-5 cDNA was generated by *EcoNI* digestion of sK4 cDNA, which removed domains 1 and 2. A linker (5'-CCCAGGGTC-3' and 5'-GGTCCCAGA-3') ligated the leader to the beginning of domain 3 in frame. K4ld1-2 cDNA was generated by a partial digestion of K4 cDNA with *BglI*. To the 3' end of this cDNA, a linker (5'-TAGATAGT-3' and 5'-GTTATCTATCAGATC-3') was added. K4ld1 cDNA was generated by a *BglI* digestion. To the 3' end of this cDNA, a linker (5'-TAGATAGT-3' and 5'-CGGATCTATCAGATC-3') was added. The chimeric G3-K43u and G3-K4c cDNAs were constructed by generating the 3'UTR and the cytoplasmic domain of ICAM-1 cDNA with *ClaI* ends by PCR using 5'-

CCATCGATGCCTGCTGGATGAGACTCCTGC-3', 5'-CCATCGATAGACTCTCACAGCA TCTGCAGC-3' and 5'-CCATCGATTGGACTATAATCATTCTGGTGC-3', 5'-CCATCG ATGAGGTGGGGCTGTTCCCTTGAG-3' oligonucleotides, respectively. The PCR products were then ligated into the unique *ClaI* site, which was converted from *BstXI* site in the 3'UTR of ICAM-2 (Ohh *et al.*, 1994) using a *ClaI* adaptor (5'-AGTATCGATTGAGCCC-3' and 5'-TCAATCGATACTGGGC-3'). All the ligated junctions between any two cDNAs and the 3' ends of the generated cDNAs were sequenced to ensure that they were in frame and the 3'ends had incorporated stop codons. The K4, K4Δ3, K4Δc3, K4ld3-5, K4ld1-2, and K4ld1 cDNAs were blunt-ligated into the *SmaI* site of a stable mammalian expression vector, pRC6 (derived from pAX114 (Kay and Humphries, 1991) with a hygromycin resistance gene driven by a thymidine kinase promoter). G3, G3-K43u, and G3-K4c cDNAs were isolated from pUC18/13 with *SalI* and ligated into the *SalI* site in pRC6. *Escherichia coli* MC1061/p3 (Yamasaki *et al.*, 1988) was used for transformation. All constructs used in this study are schematically shown in Fig. 10.

Transfection and isolation of fibroblast L cells. All cDNAs in expression vectors were transfected into ICAM-1 and -2 negative L cells by the calcium phosphate method (Sambrook *et al.*, 1989). Transfectants were isolated under hygromycin selection (250 μg/ml, Calbiochem, La Jolla, CA). The surface expression of ICAM-1 and -2 was tested by flow cytometry. Briefly, K4, K4Δ3, and K4Δc3 transfected L cells were harvested with PBS containing 2.5 mM EDTA and then stained with YN1/1.7 anti-ICAM-1 hybridoma supernatant (Horley *et al.*, 1989) containing 0.1% sodium azide and goat anti-rat Ig-FITC as a secondary antibody (Cooper Biomedical, West Chester, PA). G3, G3-K4c, and G3-K43u transfected L cells were stained with rat anti-mouse ICAM-2 mAb (PharMingen, San Diego, CA) and goat anti-rat Ig-FITC. L cells transfected with cDNA constructs incapable of surface expression (*ie.*, sK4, K4ld3-5, K4ld1-2, and K4ld1 cDNAs) were detected by a standard Northern analysis (Ohh *et al.*, 1994).

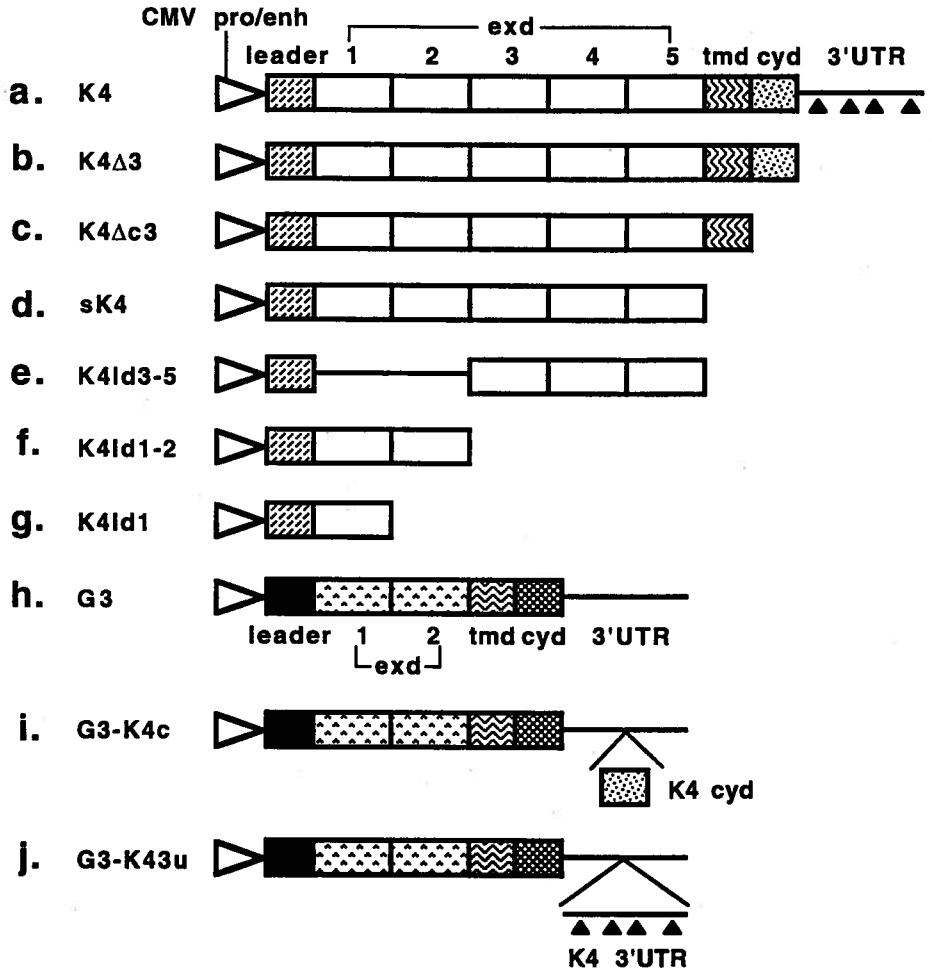


Fig. 10 Schematic diagram of the expression vectors. **a.** K4; full-length ICAM-1 expression vector. **b.** K4Δ3; ICAM-1 without the 3'UTR. **c.** K4Δc3; ICAM-1 without the cytoplasmic domain and the 3'UTR. **d.** sK4; soluble ICAM-1 without the 3'UTR, cytoplasmic and transmembrane domains. **e.** K4ld3-5; soluble ICAM-1 without domains 1 and 2. **f.** K4ld1-2; soluble ICAM-1 without domains 3 to 5. **g.** K4ld1; soluble ICAM-1 without domains 2 to 5. **h.** G3; full-length ICAM-2 expression vector. **i.** G3-K4c; cytoplasmic domain of ICAM-1 incorporated into ICAM-2 3'UTR. **j.** G3-K43u; 3'UTR of ICAM-1 incorporated into ICAM-2 3'UTR. Open triangle represents CMV promoter/enhancer; Dashed square, ICAM-1 leader sequence; Open rectangles, . ICAM-1 extracellular domains (exd); Vertical wavy square, ICAM-1 transmembrane domain (tmd); Dotted square, ICAM-1 cytoplasmic domain (cyd); Solid triangles, AUUUA pentamers within ICAM-1 3'UTR; Solid square, ICAM-2 leader sequence; Spotted rectangles, ICAM-2 extracellular domains; Horizontal wavy square, ICAM-2 transmembrane domain; Checkered square, ICAM-2 cytoplasmic domain.

ICAM-1 induction by IFN-γ or PMA. 1.5×10^6 transfected L cells were treated with or without murine IFN-γ (100 u/ml; Boehringer Mannheim, Laval, PQ) or PMA (10 ng/ml; Sigma, St. Louis, MO) and incubated at 37°C for increasing periods of time. The level of mRNA was then determined by Northern blot analysis. The bands on the autoradiogram were integrated with a densitometer and normalized to the signal of the control β-actin probe or to the constitutively expressed rRNA gene. The maximum intensity was then arbitrarily set at 100% mRNA level as a point of reference.

mRNA half-life determination. 1.5×10^6 transfected L cells were treated with actinomycin D (10 μg/ml; GIBCO, Grand Island, NY) for increasing periods of time, and the level of mRNA was determined by Northern blot analysis. The integrated band values as determined by densitometry were normalized to the constitutively expressed rRNA gene, and then the 100% mRNA level was set at time 0.

RNA preparation and Northern blot analysis. Total RNA preparation, as described by Chomczynski and Sacchi (1987) with minor modifications, and Northern blot analysis were performed as previously described (Ohh *et al.*, 1994; see p45, chapter II).

3.4 RESULTS

IFN-γ- and PMA-induced accumulation of ICAM-1 deletion-mutant mRNAs. A range of deletion-mutants of ICAM-1 cDNA shown in Fig. 10a-g were transfected into murine fibroblast L cells, which do not express endogenous ICAM-1. Upregulation of the level of ICAM-1 mRNA following the treatment of these cells with IFN-γ and PMA was then examined. IFN-γ treatment of the transfectants only induced the accumulation of K4 and K4Δ 3 mRNAs, but not the other mutant ICAM-1 mRNAs (Fig. 11a). Of particular interest is the striking difference between the cells transfected with K4Δ3 and those with K4Δc3. The latter were not responsive to IFN-γ whereas the former showed significant upregulation of ICAM-1

mRNA upon IFN-γ treatment. The only difference between these two mutant ICAM-1 mRNAs is the region encoding the cytoplasmic domain. This suggests that the region of 87 nt within ICAM-1 mRNA encoding the cytoplasmic domain contains IFN-γ-responsive element(s). On the other hand, PMA-induced accumulation of ICAM-1 transcripts was dependent on the 3'UTR since removal of this region rendered ICAM-1 mRNA unresponsive to PMA treatment (Fig. 11b). Hence, it seems likely that ICAM-1 mRNA contains at least two distinct elements which are responsive to specific cell activation signals.

PMA-induced stabilization of ICAM-1 mRNA requires 3'UTR . To further define the role of the 3'UTR of ICAM-1 mRNA in the upregulation of ICAM-1 mRNA by PMA , we generated a chimeric cDNA (G3-K43u, see Fig. 10j) consisting of the full-length ICAM-2 cDNA (G3) and ICAM-1 3'UTR. ICAM-2 gene expression is constitutive and unresponsive to various inflammatory cytokines, including IFN-γ and PMA (Staunton *et al.*, 1989; de Fougerolles *et al.*, 1991). As expected, G3 mRNA levels remained unaffected by PMA, while the chimeric G3-K43u mRNA accumulated upon PMA treatment (Fig. 12a). Furthermore, half-life analysis demonstrated that G3 mRNA had a long half-life which remained unaffected by PMA treatment, whereas G3-K43u mRNA had a relatively short half-life and was significantly stabilized by PMA stimulation (Fig. 12b). These results indicate that the 3'UTR of ICAM-1 contains destabilizing elements and PMA induces stabilization of ICAM-1 mRNA through a mechanism that acts on the ICAM-1 3'UTR. In fact, the 3'UTR of both human and murine ICAM-1 contains multiple AUUUA sequences which have been shown to act as destabilizing elements in numerous cytokine and proto-oncogene mRNAs, including GM-CSF, *c-fos*, *c-myc*, and *c-myb* (Sachs, 1993; Jackson, 1993).

Region of ICAM-1 mRNA encoding the cytoplasmic domain is responsive to IFN-γ. We also examined the region of ICAM-1 mRNA encoding the cytoplasmic domain for its responsiveness to IFN-γ. We transplanted this portion of gene onto ICAM-2 cDNA to generate a chimeric cDNA (G3-K4c; see Fig. 10i). IFN-γ treatment did not affect the level of G3 mRNA (Fig. 13a) in the transfected L cells. However, the chimeric G3-K4c mRNA was

a

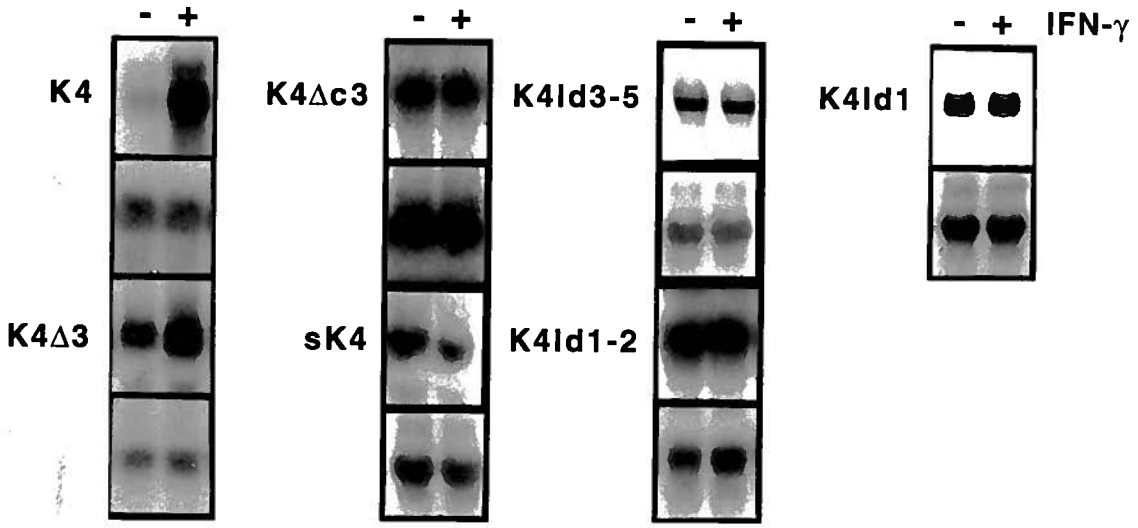
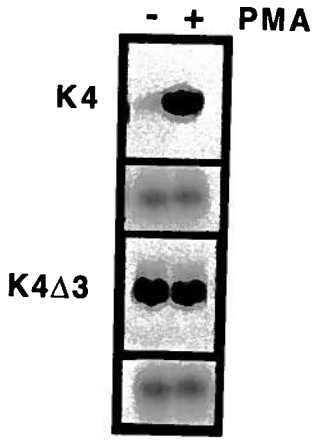


Fig. 11 Induction of various forms of ICAM-1 mRNA by IFN-γ and PMA. L cells transfected with the various forms of ICAM-1 cDNA shown in Fig. 10 were treated with (+) or without (−) IFN-γ (**a**) or PMA (**b**) for 6 h, and then subjected to Northern blot analysis. The second and fourth panels from the top represent the corresponding signals of the control β-actin. The data presented are representative of two independent experiments.

b



a

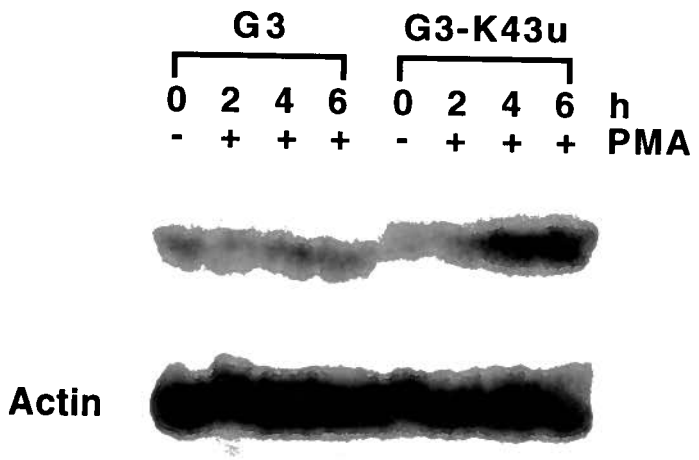
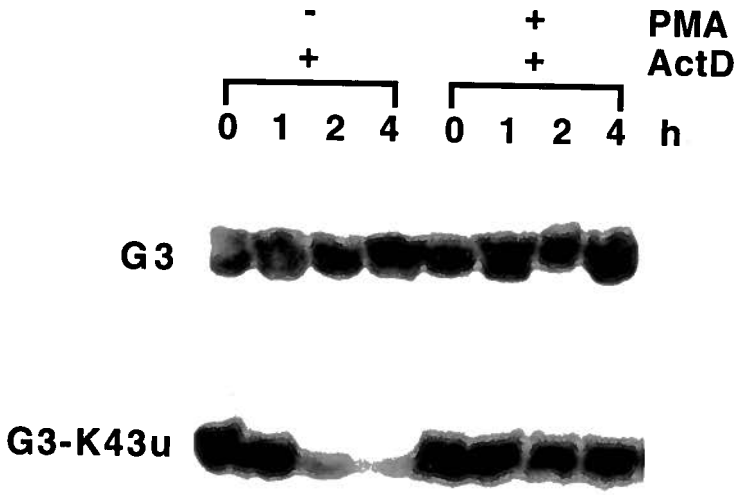


Fig. 12 Posttranscriptional effects of PMA on ICAM-2 and ICAM-2/1 chimeric mRNAs. **a.** Differential upregulation of G3 and G3-K43u mRNAs by PMA. L cells transfected with G3 and G3-K43u cDNAs were treated with PMA for the increasing periods of time, and then detected by Northern blot analysis. The bottom panel represents the corresponding signals of the control β-actin. **b.** Half-life analysis of G3 and G3-K43u mRNAs detected by Northern blot hybridization. Both L cell transfectants were treated with PMA for 2 h and then actinomycin D (ActD) was added for the indicated times. The data presented are representative of two independent experiments. The chimeric cDNAs are described in Fig. 10.

b



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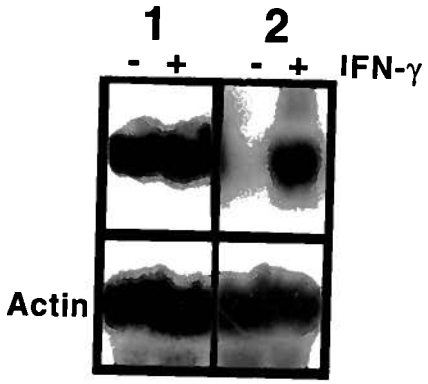
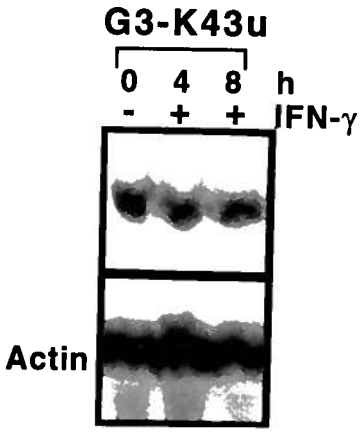


Fig. 13 Posttranscriptional effects of IFN-γ on ICAM-2 and ICAM-2/1 chimeric mRNAs. **a.** Differential upregulation of G3 and G3-K4c mRNAs by IFN-γ. L cells transfected with G3 (panel 1) and G3-K4c (panel 2) cDNAs were treated with (+) or without (–) IFN-γ for 6 h, and then detected by Northern blot analysis. The bottom panels represent the corresponding signals of the control β-actin. **b.** Effect of IFN-γ on G3-K43u mRNA accumulation. L cells transfected with G3-K43u cDNA were treated with IFN-γ for the increasing periods of time and then detected by Northern blot analysis. The bottom panel represents the corresponding signals of the control β-actin. **c.** Half-life analysis of K4Δ3, K4Δc3, and G3-K4c mRNAs detected by Northern blot hybridization. L cell transfectants were treated with IFN-γ for 2 h and then actinomycin D (ActD) was added for the indicated times. The data presented are representative of two independent experiments. The chimeric cDNAs used for the transfection are described in Fig. 10.

b



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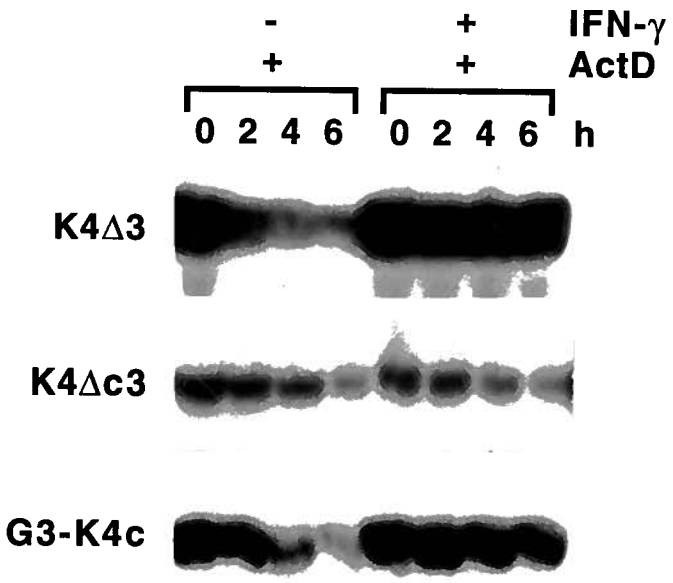

markedly induced upon IFN-γ treatment (Fig. 13a). G3-K43u mRNA that was responsive to PMA was not affected by IFN-γ treatment (Fig. 13b). Half-life analysis showed that G3-K4c mRNA was relatively unstable but was stabilized by IFN-γ treatment (Fig. 13c). Furthermore, K4Δ3 mRNA had a shorter half-life than K4Δc3 mRNA, and only the stability of K4Δ3 mRNA was increased upon IFN-γ treatment. The difference between the two mRNAs is the absence of the cytoplasmic encoding region in the K4Δc3 mRNA. These results, taken together, suggest that the region of ICAM-1 mRNA encoding the cytoplasmic domain contains destabilizing element(s) responsive to IFN-γ.

3.5 DISCUSSION

The results presented above indicate that IFN-γ and PMA stabilize and upregulate ICAM-1 mRNA by acting on two distinct destabilizing elements in ICAM-1 mRNA. The IFN-γ - responsive element(s) is found within the region encoding the cytoplasmic domain. This 87 nt region is necessary for the stabilization and upregulation of ICAM-1 mRNA by IFN-γ. When fused with ICAM-2 mRNA, it is also sufficient to make stable ICAM-2 mRNA unstable and responsive to IFN-γ in mRNA stabilization. On the other hand, the 3'UTR of ICAM-1 mRNA is necessary and sufficient for the effect of PMA to stabilize and induce upregulation of mRNA. This effect of ICAM-1 3'UTR is to be expected, because it contains multiple AUUUA sequences which are known to destabilize many other mRNAs. Our findings with IFN-γ are somewhat unexpected, because the region of ICAM-1 mRNA responsive to IFN-γ encoding the cytoplasmic domain is only 87 nt long and does not contain AU-rich sequences or other readily recognizable unique sequences.

The ability of IFN-γ to upregulate ICAM-1 mRNA and cell surface expression of the ICAM-1 protein has been well documented (Dustin *et al.*, 1986; Pober *et al.*, 1986; Rothlein *et al.*, 1988; Degitz *et al.*, 1991). However, previous studies mainly focused on the ability of

IFN-γ to induce transcription of the ICAM-1 gene (Degitz *et al.*, 1991; Voraberger *et al.*, 1991; Wertheimer *et al.*, 1992). We have previously reported that IFN-γ may have an additional effect, which is to stabilize otherwise unstable ICAM-1 mRNA (Ohh *et al.*, 1994). This effect of IFN-γ may be particularly relevant for the upregulation of ICAM-1 by IFN-γ on some cells, including endothelial cells that constitutively express low levels of ICAM-1. It is likely that this low basal level of ICAM-1 expression is maintained, at least in part, by a process that reduces the half-life of ICAM-1 mRNA. As our present study has demonstrated, at least two distinct mechanisms can stabilize and upregulate ICAM-1 mRNA levels. Posttranscriptional control of ICAM-1 gene expression may thus be an important mechanism regulating the expression of ICAM-1 on cells that constitutively express low levels of ICAM-1. Such a mechanism might then prove to play a key role in regulating the ability of leukocytes to first adhere to endothelial cells and then subsequently transmigrate into inflamed tissues.

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IV

REGULATION OF ICAM-1 mRNA STABILITY BY CYCLOHEXIMIDE: ROLE OF SERINE/THREONINE PHOSPHORYLATION AND PROTEIN SYNTHESIS

4.1 ABSTRACT

Cycloheximide is a protein synthesis inhibitor that superinduces the expression of many genes by preventing the degradation of otherwise labile mRNAs. In some genes this depends on the presence of the AUUUA destabilizing multimers in the 3'UTR. The effect of cycloheximide on the expression of the murine ICAM-1 gene in several cell lines including A20 (B cell lymphoma), T28 (T cell hybridoma), P388D1 (monocytic cell), SVEC4-10 (lymphoid endothelial cell), and ICAM-1-transfected murine fibroblast L cells was examined. Cycloheximide was able to dramatically increase the accumulation of ICAM-1 mRNA in all the cell lines examined except T28, and this seemed to be due to the stabilization of the ICAM-1 mRNA as indicated by mRNA half-life analyses. To determine whether these effects were dependent on the 3'UTR containing AUUUA sequences, L cells were transfected with either full-length ICAM-1 cDNA or a truncated form lacking AUUUA sequences in their 3'UTR (ICAM-1Δ3). There was no discernible difference in the effect of cycloheximide on ICAM-1 mRNA accumulation or half-life between the two types of tranfected cells. The effect of cycloheximide on ICAM-1 mRNA was markedly suppressed by serine/threonine (ser/thr) kinase inhibitors, H-7 and staurosporine, whereas the ser/thr phosphatase inhibitor, okadaic acid, augmented the cycloheximide effect. Inhibitors of protein tyrosine kinases and phosphatases had no effect. Unexpectedly, the level of cell surface ICAM-1 as well as *de novo* synthesis of ICAM-1 in SVEC4-10 and the ICAM-1-transfected L cells were also upregulated by cycloheximide whereas the overall protein synthesis in these cells were profoundly

inhibited, suggesting that ICAM-1 protein synthesis in these cells escapes the translational inhibition by cycloheximide. These results suggest that the stabilization of ICAM-1 mRNA by cycloheximide is independent of its translational inhibition and that ser/thr phosphorylation of unidentified protein(s) seems to play a crucial role in mediating this ICAM-1 mRNA stabilizing effect.

4.2 INTRODUCTION

ICAM-1 expression is dramatically increased at sites of inflammation (Rothlein *et al.*, 1986; Dustin *et al.*,1986; Pober *et al.*, 1986), thereby providing important means of regulating cell-cell interactions and the enhanced expression of ICAM-1 on venule endothelium is known to facilitate the adhesion and subsequent trans-endothelial migration of leukocytes bearing LFA-1 or Mac-1 into inflammatory tissues (for review, see Wawryk *et al.*, 1989; Smith *et al.*, 1989), as well as appropriate interaction of lymphocytes with cells expressing targeted antigens (Dougherty *et al.*, 1988; Wawryk *et al.*, 1989; Springer, 1990). However, despite the obvious importance of inducible ICAM-1 expression in numerous immunologic responses, very little is known about the intracellular mechanisms controlling its expression. Although there is an increasing body of evidence that mRNA turnover plays an important role in regulating gene expression. For example, the 3'UTRs of both human and murine ICAM-1 mRNAs contain several AUUUA sequences, implicated in the decay of a number of short-lived mRNAs of this class (Sachs, 1993; Shaw and Kamen, 1986; Schuler and Cole, 1988), although an involvement of AUUUA sequences in the 3'UTR of ICAM-1 mRNA in the control of its turnover has not been determined.

Cycloheximide is widely used to stabilize labile mRNAs. Because of its ability to inhibit peptidyl transferase, this effect of cycloheximide implies that the stability of mRNA is associated with protein synthesis. For example, almost all mRNAs in yeast are stabilized by

cycloheximide (Herrick *et al.*, 1990). Similarly, the degradation of mRNAs for histone, β-tubulin, transferrin receptor, certain proto-oncogenes, and various lymphokines is dramatically reduced in the presence of translational inhibitors (for reviews, see Jackson, 1993 and Sachs, 1993). These observations suggest that most mRNAs need to be translated to be degraded. However, it is also possible that mRNAs are degraded by a unique class of highly labile proteins that are no longer synthesized but continue to be degraded upon cycloheximide treatment. The mechanism by which labile mRNAs are stabilized by cycloheximide has not yet been elucidated.

We report here that cycloheximide stabilizes otherwise labile ICAM-1 mRNA and that this effect is independent of the 3'UTR which contains multiple AUUUA sequences. Inhibitors of ser/thr kinases as well as those for phosphatases have profound effects on the induction and stabilization of ICAM-1 mRNA by cycloheximide. Furthermore, in some cells ICAM-1 synthesis is increased by cycloheximide treatment whereas the overall protein synthesis is strongly inhibited, indicating that the stabilization of ICAM-1 mRNA can be obtained whether or not its translation is inhibited.

4.3 MATERIALS AND METHODS

Cell culture. The murine B cell lymphoma A20, T cell hybridoma T28, monocytic cell line P388D1, lymph node endothelial cell line SVEC4-10 (O'Connell and Edidin, 1990), and the fibroblast L cells were cultured in DMEM (GIBCO, Grand Island, NY) containing 10% FCS and antibiotics.

Expression vectors and transfection into L cells. Expression vectors were constructed using standard recombinant DNA techniques (Sambrook *et al.*, 1989). Briefly, K4-1.1 ICAM-1 cDNA (Horley *et al.*, 1989) was partially digested with *PstI* to remove its 3'UTR. The soluble G3-1.1 ICAM-2 (sICAM-2) cDNA (Ohh *et al.*, 1994) in pBST was cut out with *XbaI*

and *NotI*. The sICAM-2, truncated (ICAM-1Δ3) and the full-length ICAM-1 cDNAs were blunt-ligated into *SmaI* site of a stable mammalian expression vector, pRC6 (derived from pAX114 (Kay and Humphries, 1991) with a hygromycin resistance gene driven by a thymidine kinase promoter). *Escherichia coli* MC1061/p3 (Yamasaki *et al.*, 1988) was used for transformation. ICAM-1, ICAM-1Δ3, and sICAM-2 cDNAs in expression vectors were transfected into ICAM-1 and -2 negative L cells by the calcium phosphate method (Sambrook *et al.*, 1989). Transfectants (L-ic-1, L-ic-1Δ3, and L-sic-2) were isolated under hygromycin selection (250 μg/ml; Calbiochem, La Jolla, CA). The surface expression of ICAM-1 was tested by flow cytometry as described below.

ICAM-1 induction by cycloheximide and phosphorylation inhibitors. 1.5×10^6 cells were pretreated for 15 min at 37°C with various phosphorylation inhibitors, including staurosporine (0.2 μM; Calbiochem), H-7 (10 μM; Calbiochem), H-89 (0.1 μM; Calbiochem), bisindolylmaleimide (0.03 μM; Calbiochem), okadaic acid (0.5 μM; Calbiochem), genistein (20 μM; Calbiochem), tyrphostein (30 μM; Calbiochem), and vanadate (50 μM; Calbiochem). Then cycloheximide (10 μg/ml; Sigma, St. Louis, MO) was added for increasing periods of time at 37°C. The level of ICAM-1 mRNA was then determined by Northern blot analysis as described below.

ICAM-1 mRNA half-life determination. 1.5×10^6 cells were treated with various phosphorylation inhibitors in combination with cycloheximide for 2 h at 37°C, and then actinomycin D (10 μg/ml, GIBCO, Grand Island, NY) was added for increasing periods of time. The level of ICAM-1 mRNA was determined by Northern blot analysis as described below.

RNA preparation and Northern blot analysis. see p45, chapter II.

Flow cytometric analysis. Cells were treated with various kinase and phosphatase inhibitors in combination with cycloheximide for 12 h. Briefly, the cells were washed with Hank's balanced salt solution containing 2% FCS (the adherent cells, SVEC4-10 and L-ic-1, were then harvested with PBS containing 2.5 mM EDTA). Cells were directly stained with

anti-ICAM-1 (YN1/1.7) -FITC mAb (Horley *et al.*, 1989) containing 0.1% sodium azide and analyzed on fluorescence-activated cell sorter (FACSort, Becton Dickinson, Mountain View, CA). Dead cells stained with propidium iodide were gated out.

Metabolic labeling and immunoprecipitation. 1.5×10^6 cells cultured in DMEM + 10% FCS were treated with cycloheximide (10 μg/ml) or puromycin (10 μM) at 37°C for various times and 2.5 h prior to cell lysis, the cells were washed with PBS and incubated in methionine-free DMEM + 10% dialyzed FCS + cycloheximide (10 μg/ml) or + puromycin (10 μM). After 30 min, 0.25 mCi/ml of [^{35}S]-methionine was added for 2 h. The cells were then washed with PBS (the adherent cells, SVEC4-10 and L-ic-1, were then harvested with PBS containing 2.5 mM EDTA) and solubilized with 0.5% NP-40 in 4°C PSB (50 mM Hepes, pH 7.4, 100 mM NaF, 10 mM NaPPi, 2 mM Na_3VO_4, 4 mM EDTA, 2 mM PMSF, 10 μg/ml leupeptin, and 2 μg/ml aprotinin). After 1 h at 4°C, the cell lysates were clarified by centrifugation for 10 min. 60 μl of the supernatant was kept as total cell lysate and to the remaining supernatant, 20 μl of YN1/1.7 anti-ICAM-1 hybridoma supernatant was added. Following 1 h of gentle agitation at 4°C, immune complexes were bound onto anti-rat Ig-coated beads, which were previously treated at 4°C for 2 h with Tris-buffered saline (TBS, 10 mM Tris-Cl, pH 8.0, 0.15 M NaCl) containing 5% skim milk powder and 5% BSA to minimize non-specific binding, at 4°C for 1.5 h, washed gently thrice in cold PSB and then boiled in SDS-sample buffer for 2 min. The total cell lysates were also boiled in SDS-sample buffer and all the samples were subjected to SDS-PAGE analysis (Sambrook *et al.*, 1989) using 7.5% polyacrylamide gels.

4.4 RESULTS

Role of 3'UTR in the induction of ICAM-1 mRNA by cycloheximide. Both human and murine ICAM-1 mRNAs contain multiple AUUUA sequences in their 3'UTRs. Treatment of various

murine cell lines, including A20 (B cell lymphoma), T28 (T cell hybridoma), P388D1 (monocytic cell), and SVEC4-10 (lymphoid endothelial cell), with cycloheximide dramatically increased the accumulation of ICAM-1 mRNA in all cell types examined except in T28 (Fig. 14). In order to investigate the role of the 3'UTR containing multiple AUUUA repeats in the upregulation of ICAM-1 mRNA level, a full-length murine ICAM-1 cDNA clone K4-1.1 and its truncated form without the 3'UTR (ICAM-1Δ3) were subcloned into the mammalian expression vector pRC6 (Fig. 15a) and then stably transfected into murine fibroblast L cells, which do not express detectable levels of ICAM-1 mRNA regardless of cycloheximide treatment. Bulk populations of L cells transfected with full-length ICAM-1 (L-ic-1) and those with ICAM-1Δ3 (L-ic-1Δ3) were established by hygromycin resistance and the cell surface expression of ICAM-1 was confirmed by flow cytometry (see below). Northern blot analysis clearly showed that upon cycloheximide treatment both ICAM-1 and ICAM-1Δ3 mRNA levels increased dramatically at a rapid rate (Fig. 15b). However, the soluble ICAM-2 (sICAM-2) mRNA was only slightly induced by cycloheximide (Fig. 15c), which demonstrates that the 3'UTR (rabbit β-globin) supplied by the vector is unresponsive to cycloheximide. Therefore, the 3'UTR of ICAM-1 containing the AUUUA multimers are not required for the induction of ICAM-1 mRNA by cycloheximide.

Ser/thr kinase and phosphatase inhibitors and cycloheximide effect. PMA has been shown to upregulate ICAM-1 mRNA level by stabilizing it (Wertheimer *et al.*, 1992), suggesting that protein kinases may be involved in the regulation of ICAM-1 mRNA expression. To examine the role of protein kinases in the effects of cycloheximide on ICAM-1 mRNA expression, L-ic-1 cells were treated overnight with PMA to downregulate PKC. However, such treatment, whether 10 or 40 ng/ml, prior to the addition of cycloheximide did not inhibit the accumulation of ICAM-1 transcripts (Fig. 16). On the other hand, treatment of cells with staurosporine, which is a general ser/thr kinase inhibitor, prior to the addition of cycloheximide completely inhibited the induction of ICAM-1 mRNA level (Fig. 16). The addition of staurosporine after the overnight treatment with PMA abolished the cycloheximide

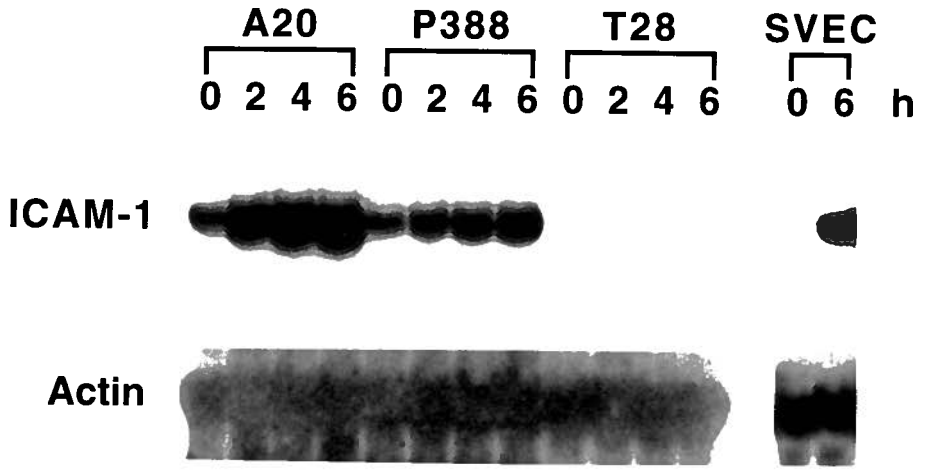

Fig. 14 Effects of cycloheximide on ICAM-1 mRNA expression. A20, P388D1, T28, and SVEC4-10 cells were treated with cycloheximide for the increasing periods of time and the ICAM-1 mRNA levels were detected by Northern blot analysis. The bottom panels represent the signals of the control β-actin. The data presented are representative of three independent experiments.

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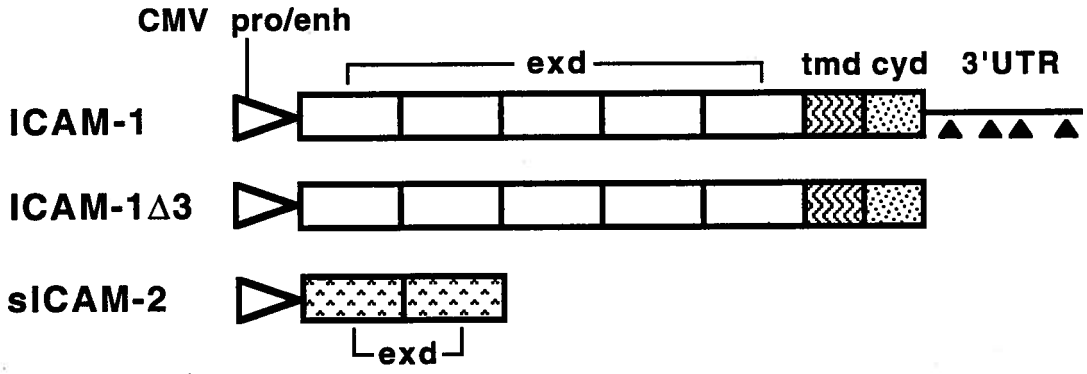
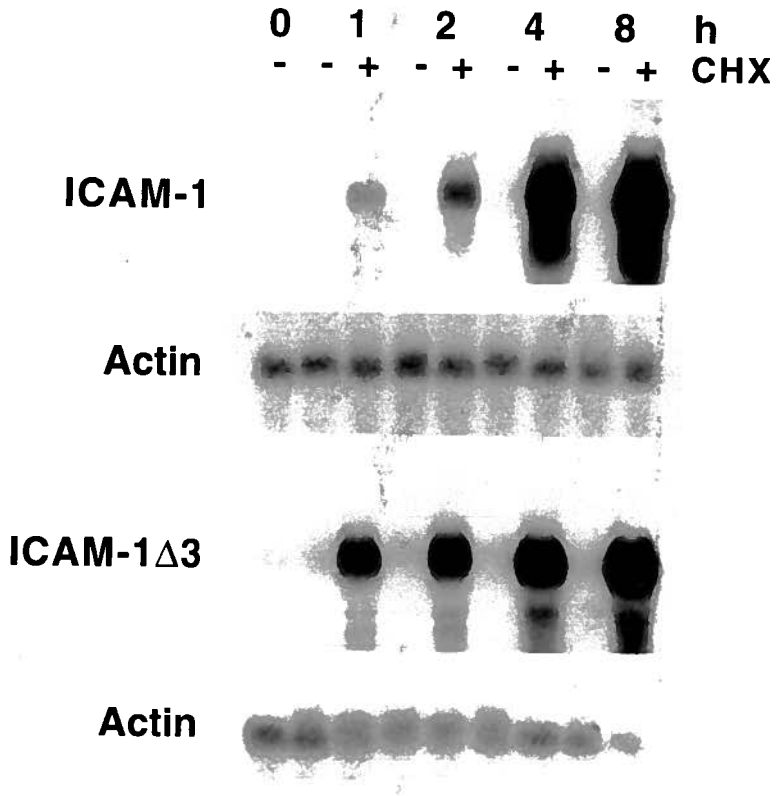


Fig. 15 Effects of cycloheximide on ICAM-1 mRNA expression in L-ic-1 and L-ic-1Δ3 cells and on ICAM-2 mRNA expression in L-sic-2 cells. **a.** Schematic diagrams of pRC6-ICAM-1, pRC6-ICAM-1Δ3, and pRC6-sICAM-2 expression vectors. Open triangle represents cytomegalovirus (CMV) promoter/enhancer (pro/enh); solid triangles, AUUUA pentamers; open rectangle, ICAM-1 extracellular domains (exd); vertical wavy square, ICAM-1 transmembrane domain (tmd); dotted square, ICAM-1 cytoplasmic domain (cyd); spotted rectangle, ICAM-2 extracellular domains. **b.** The induction of ICAM-1 and ICAM-1Δ3 mRNA levels in L-ic-1 and L-ic-1Δ3 cells stimulated with (+) or without (–) cycloheximide (CHX) for the indicated times, as detected by Northern blot analysis. The second and fourth panels from the top represent the corresponding signals of the control β-actin. The data presented are representative of three independent experiments. **c.** The induction of sICAM-2 mRNA levels in L-sic-2 cells stimulated with cycloheximide (CHX) for the indicated times, as detected by Northern blot analysis. The second panel represents the corresponding signals of the control β-actin. The data presented are representative of two independent experiments.

b



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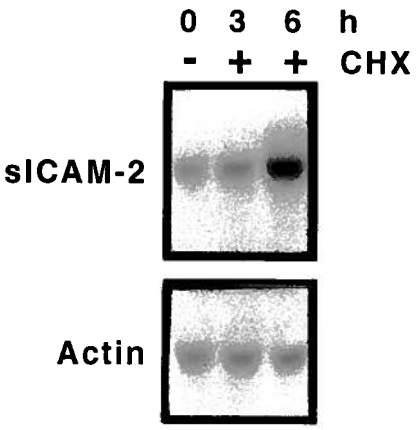

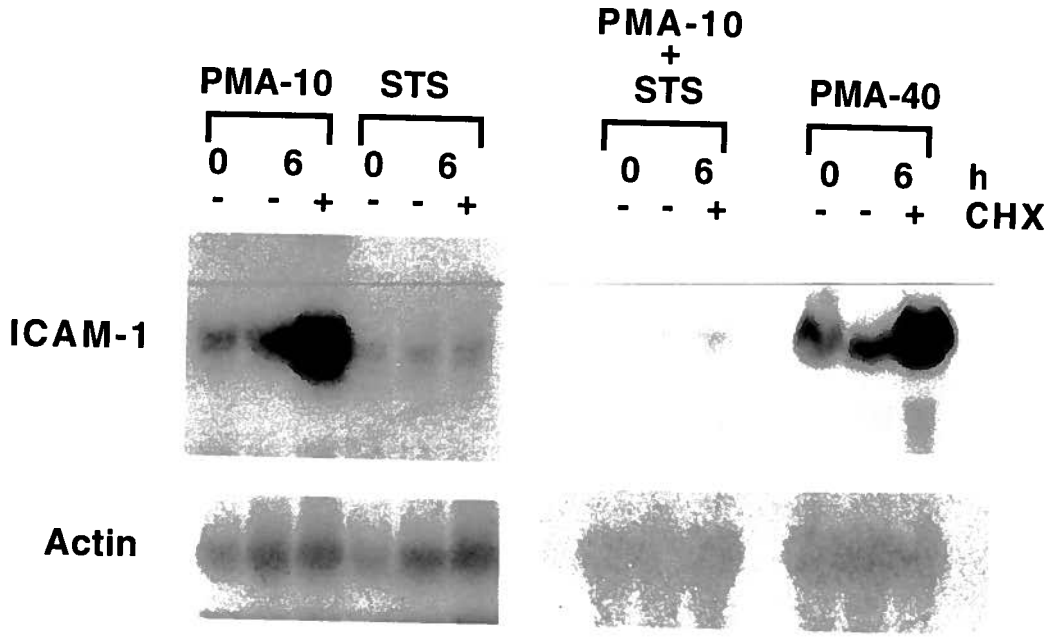

Fig. 16 Effects of PMA over-stimulation and staurosporine on ICAM-1 mRNA accumulation induced by cycloheximide. Cells were treated with PMA (either 10 or 40 ng/ml; PMA-10 and PMA-40, respectively) for 72 h or staurosporine alone for 15 min (STS) or PMA for 72 h followed by staurosporine for 15 min (PMA-10 + STS), and then incubated in the presence of cycloheximide (CHX) for 6 h. The accumulation of ICAM-1 transcripts was determined by Northern blot analysis. The bottom panels represent the signals of the control β-actin. The data presented are representative of five independent experiments performed on L-ic-1, L-ic-1 Δ3, SVEC4-10, P388D1, and A20 cells.

effect on ICAM-1 mRNA induction (Fig. 16). These results suggest that the effect of staurosporine is not due to the inhibition of PKC but is due to the inhibition of other ser/thr kinase(s). Consistent with the staurosporine result, H-7, another ser/thr kinase inhibitor, strongly inhibited the effect of cycloheximide (Fig. 17a). In contrast, treatment with the ser/thr phosphatase inhibitor, okadaic acid, alone increased ICAM-1 mRNA level, and in combination with cycloheximide augmented the cycloheximide effect (Fig. 17a,b). The protein tyrosine kinase inhibitor, genistein, and tyrosine phosphatase inhibitor, vanadate, had no discernible effect on ICAM-1 mRNA accumulation induced by cycloheximide (Fig. 17a). Tests were also performed using tyrphostein instead of genistein and gave similar results. In agreement with the PMA over-stimulation results shown in Fig. 16, bisindolylmaleimide, a highly selective inhibitor of PKC, or H-89, a potent inhibitor of cAMP-dependent protein kinase, was also unable to inhibit the cycloheximide effect (Fig. 17b). These results, taken together, suggest that cycloheximide induces the accumulation of ICAM-1 mRNA through a ser/thr phosphorylation-dependent pathway.

Half-life analysis. Although ICAM-1 mRNA has a short half-life (Fig. 18a,b), treatment with cycloheximide dramatically prolonged the half-life to far greater than 2 h (Fig. 18a). Treatment with the ser/thr kinase inhibitors, staurosporine and H-7, alone had a negligible effect on the turnover rate (Fig. 18a). However, the cycloheximide effect was efficiently inhibited by these ser/thr kinase inhibitors (Fig. 18a). Treatment with the ser/thr phosphatase inhibitor, okadaic acid, alone extended the half-life to far greater than 2 h (Fig. 18b). Concordant with the induction analysis, the half-life analysis strongly suggests that cycloheximide stabilizes the otherwise labile ICAM-1 mRNA through a ser/thr phosphorylation-dependent pathway. The induction and half-life analyses were also performed in duplicate on A20, P388D1, and SVEC4-10 cells and they all gave similar and consistent results. Furthermore, sICAM-2 mRNA had a relatively long half-life (Fig. 18c) and as expected from the induction analysis, treatment of L-sic-2 cells with cycloheximide had a negligible effect on the sICAM-2 mRNA turnover rate.

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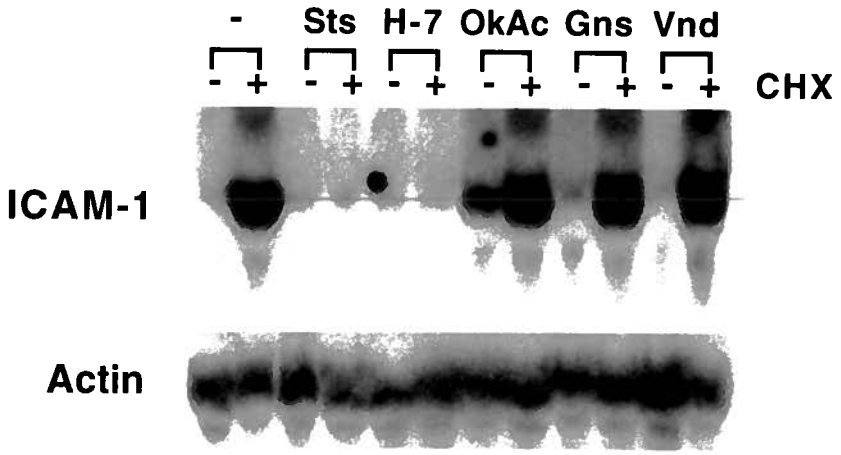

Fig. 17 Effects of phosphorylation inhibitors on ICAM-1 mRNA accumulation by cycloheximide. **a.** Cells were first treated with various phosphorylation inhibitors (Sts represents staurosporine; H-7,H-7; OkAc, okadaic acid; Gns, genistein; Vnd, vanadate; –, no phosphorylation inhibitor added) for 15 min and then the cells were treated with (+) or without (–) cycloheximide (CHX) for 6 h. **b.** Cells were first treated with various phosphorylation inhibitors (BIM represents bisindolylmaleimide; H-89,H-89) for 15 min and then the cells were treated with (+) or without (–) cycloheximide for 6 h. The accumulation of ICAM-1 transcripts in *a* and *b* was determined by Northern blot analysis. The bottom panels represent the signals of the control β-actin. The data presented are representative of five independent experiments performed on L-ic-1, L-ic-1Δ3, SVEC4-10, P388D1, and A20 cells.

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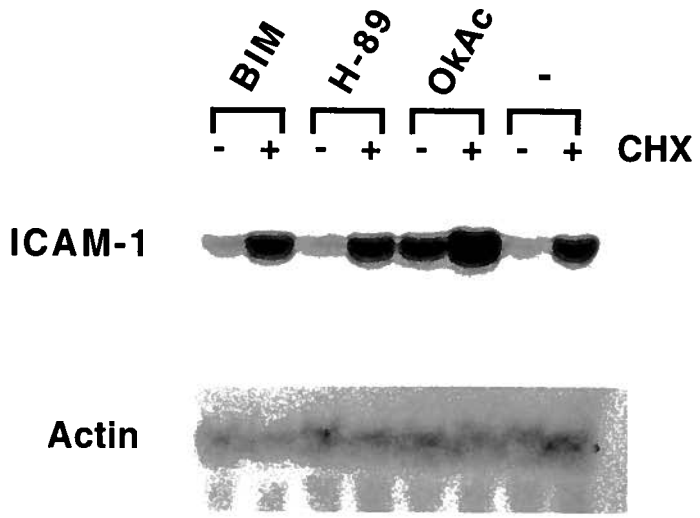

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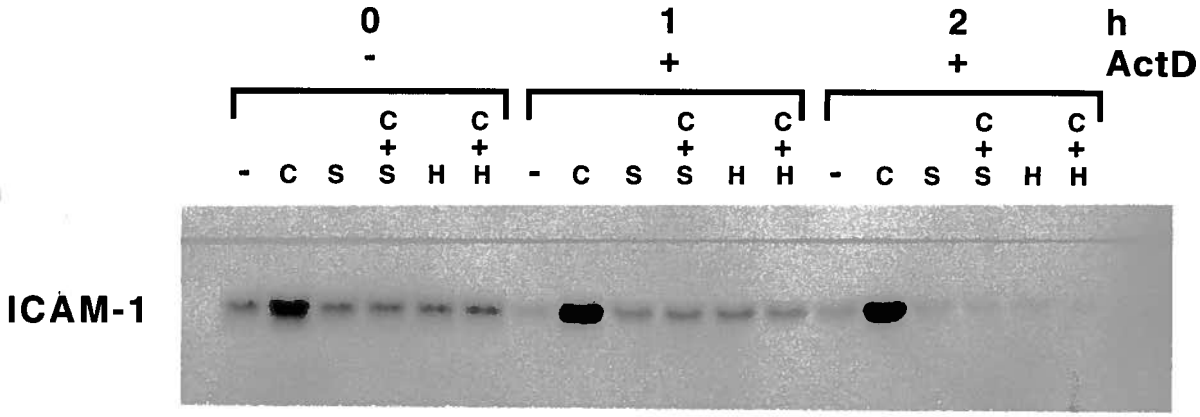
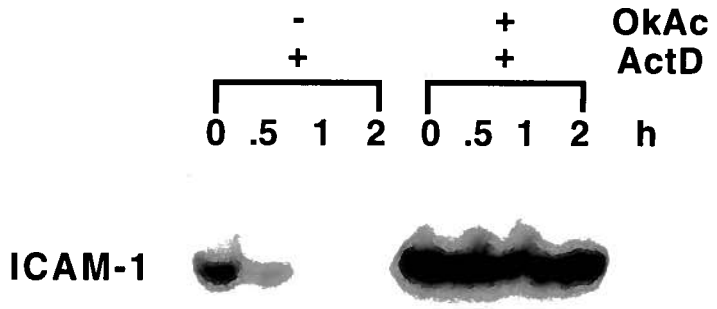


Fig. 18 ICAM-1 and ICAM-2 mRNA half-life analysis. **a.** Cells were treated with phosphorylation inhibitors (S represents staurosporine; H, H-7; –, no inhibitor added) for 15 min, followed by stimulation with or without cycloheximide (C) for 2 h. Actinomycin D (ActD) was then added for the indicated times. **b.** Cells were treated with (+) or without (–) okadaic acid (OkAc) for 2 h and then actinomycin D was added for the indicated times. ICAM-1 mRNA levels in *a* and *b* were determined by Northern blot analysis. Equalized loading of total RNA in each lane was confirmed by the ethidium bromide staining of the constitutively expressed rRNA gene. The data presented are representative of five independent experiments performed on L-ic-1, L-ic-1Δ3, SVEC4-10, P388D1, and A20 cells. **c.** L-sic-2 cells were treated with cycloheximide (CHX) for 2 h and then actinomycin D was added for the indicated times. sICAM-2 mRNA levels were measured by Northern blot analysis. Equalized loading of total RNA in each lane was confirmed by the ethidium bromide staining of the constitutively expressed rRNA gene. The data presented are representative of two independent experiments.

b



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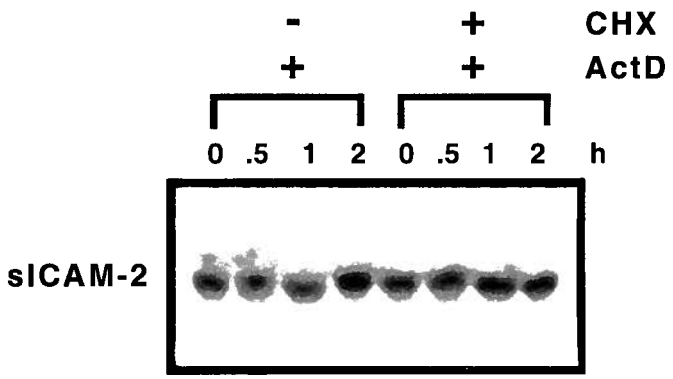


Flow cytometric analysis of cell surface ICAM-1 expression. Since the primary effect of cycloheximide is thought to be protein synthesis inhibition, we examined the effect of cycloheximide on ICAM-1 protein expression on the cell surface by flow cytometry in the presence or absence of various phosphorylation inhibitors. Contrary to the expected reduction in ICAM-1 expression, the addition of cycloheximide significantly increased the surface expression of ICAM-1 on L-ic-1 cells (Fig. 19b). Untransfected L cells did not express ICAM-1 on the cell surface regardless of cycloheximide treatment (Fig. 19a). The treatment of the transfected L cells with the tyrosine kinase inhibitors (genistein and tyrphostein), tyrosine phosphatase inhibitor (vanadate), general ser/thr kinase inhibitors (H-7 and staurosporine), the inhibitors of PKC and PKA (bisindolylmaleimide and H-89, respectively) had no effect on the expression of ICAM-1 (Fig. 19c). Another translational inhibitor, puromycin, alone also had no effect, indicating that the effect of cycloheximide is not general to all protein synthesis inhibitors (Fig. 19c). Okadaic acid alone was able to increase the expression of ICAM-1 (Fig. 19d). The upregulation of cell surface ICAM-1 expression by cycloheximide was markedly reduced by the addition of staurosporine or H-7 (Fig. 19e,f). On the other hand, other inhibitors had no effect on the upregulation of cell surface ICAM-1 expression by cycloheximide (Fig. 19g,h). These results are consistent with the observations made at the mRNA level, and strongly suggest again that cycloheximide upregulates the expression of ICAM-1 through a ser/thr phosphorylation-dependent pathway. However, A20, P388D1, and T28 cells did not show any discernible change in the expression of ICAM-1 following cycloheximide treatment (Fig. 20), suggesting a tissue-specific effect of cycloheximide.

Measurement of de novo ICAM-1 protein synthesis in the presence of cycloheximide. Upregulation of ICAM-1 expression on the cell surface by cycloheximide treatment (described above) might be explained by an ineffective inhibition of protein synthesis in the cell lines tested. Therefore, ICAM-1 protein synthesis in cycloheximide treated cells was determined. A20, SVEC4-10, and L-ic-1 cells were treated with cycloheximide for increasing periods of time and then labeled with [^{35}S]-methionine for 2 h. ICAM-1 proteins were

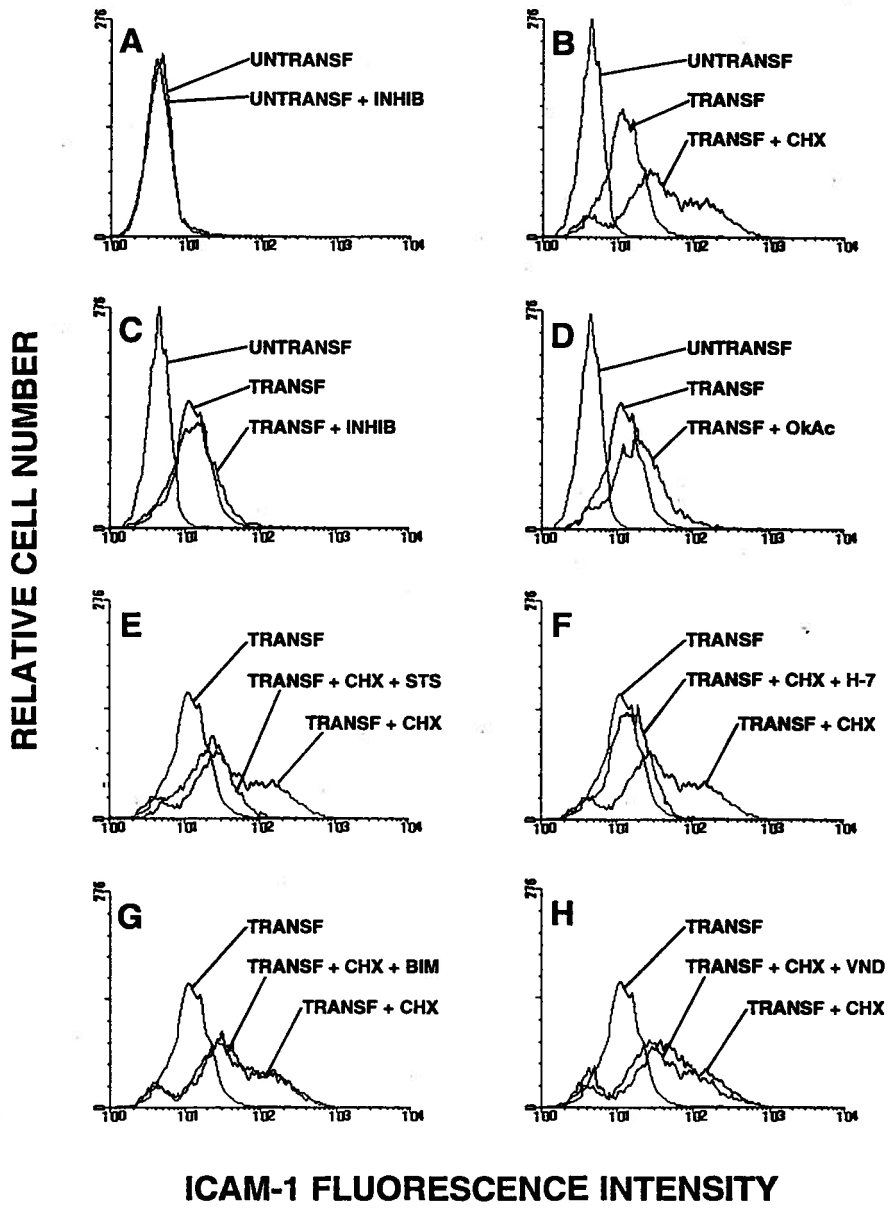


Fig. 19 Flow cytometric analysis of ICAM-1 cell surface expression. Cells were treated with kinase and phosphatase inhibitors in combination with cycloheximide for 12 h, then stained with YN1/1.7-FITC mAb and analyzed by flow cytometry. **A.** Effects of inhibitors including staurosporine, H-7, bisindolylmaleimide, H-89, okadaic acid, genistein, tyrphostein, vanadate, puromycin, and cycloheximide on untransfected L cells (UNTRANSF + INHIB). Representative profile. **B.** Cycloheximide treatment of L-ic-1 (TRANSF + CHX). **C.** Inhibitor (as in A except okadaic acid) treatment of L-ic-1 (TRANSF + INHIB). Representative profile. **D.** Okadaic acid treatment of L-ic-1 (TRANSF + OkAc). **E.** Treatment with cycloheximide alone (TRANSF + CHX) or in combination with staurosporine (TRANSF + CHX + STS). **F.** As in E except with H-7. **G.** as in E except with bisindolylmaleimide (BIM). **H.** As in E except with vanadate (VND). G and H also represent the effects of genistein, tyrphostein, and H-89. The data presented are representative of three independent experiments performed on L-ic-1, L-ic-1Δ3, and SVEC4-10 cells.

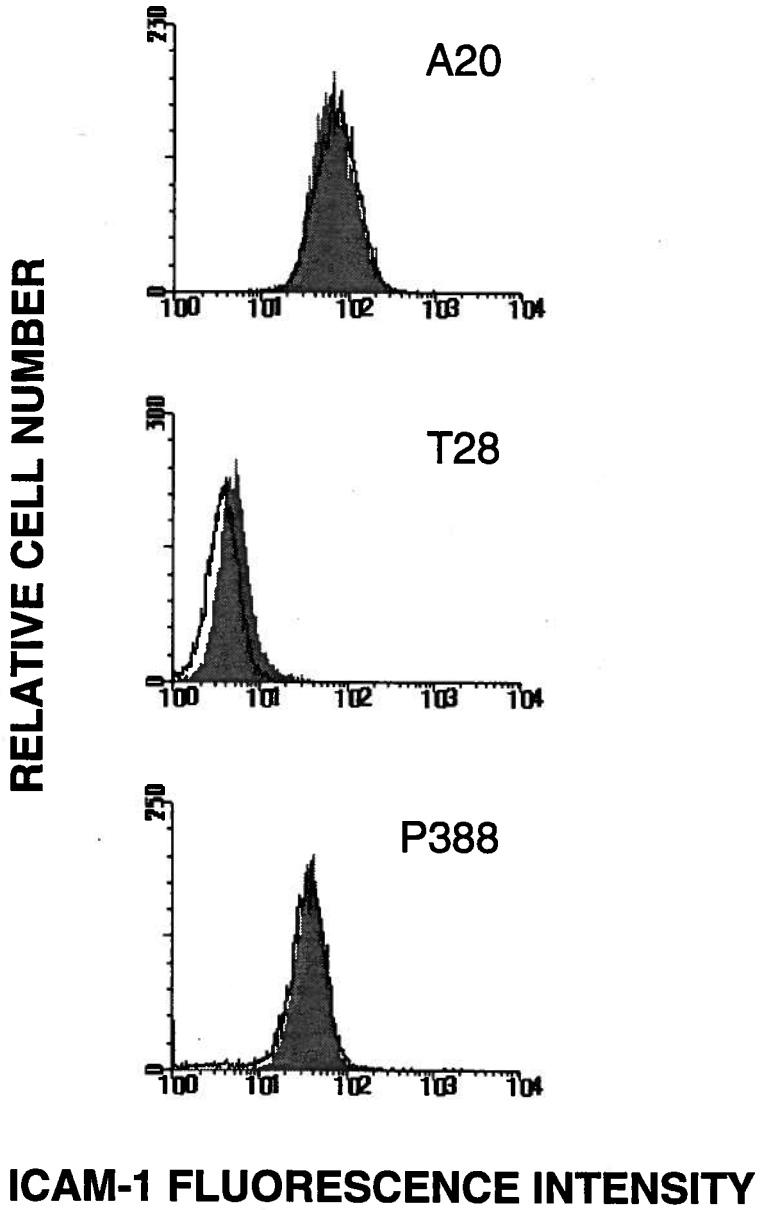


Fig. 20 Flow cytometric analysis of the effects of cycloheximide on ICAM-1 cell surface expression on A20, P388D1, and T28 cells. Cells were treated with cycloheximide for 12 h, then stained with YN1/1.7-FITC mAb and analyzed by flow cytometry. Shaded histograms represent the cell surface expression of ICAM-1 without the cycloheximide treatment; lined histograms, ICAM-1 cell surface expression with the cycloheximide treatment.

immunoprecipitated and analyzed by SDS-PAGE. It was clear from the total cell lysate fraction of each cell type that cycloheximide almost completely inhibited the overall translational capacity of the cell (Fig. 21a). Concordantly, ICAM-1 protein synthesis was significantly reduced in A20 cells (Fig. 21a). The band around 90 kDa at time 0 for the A20 cells was most likely ICAM-1 protein since a negative control mAb, YE1/48, did not immunoprecipitate a protein of similar size (Fig. 21b). Furthermore, a puromycin-treated total cell lysate fraction also showed marked inhibition of the overall translational capacity of the cell, as well as diminished ICAM-1 protein level (Fig. 21c). However, cycloheximide did not diminish but rather increased ICAM-1 protein synthesis in SVEC4-10 and the L-ic-1 cells (Fig. 21a). Hence, in agreement with the flow cytometric data, these metabolic labeling experiments show that ICAM-1 protein synthesis in the SVEC4-10 endothelial cells and the L-ic-1 fibroblast cells "escapes" the translational inhibitory effect of cycloheximide. The ser/thr kinase and phosphatase inhibitors had no measurable effect on protein synthesis and did not influence the translational inhibition by cycloheximide (Fig. 21d).

4.5 DISCUSSION

This series of experiments demonstrate first, that cycloheximide stabilizes and superinduces ICAM-1 mRNA, but that this effect does not require AUUUA repeats in the 3'UTR; secondly, that the ser/thr kinase inhibitors, staurosporine and H7, abrogate the effect of cycloheximide whereas the ser/thr phosphatase inhibitor, okadaic acid, alone stabilizes ICAM-1 mRNA and also augments the ability of cycloheximide treatment to stabilize ICAM-1 mRNA; and finally that although cycloheximide inhibits the overall level of protein synthesis, it can enhance the synthesis of ICAM-1 protein and its associated expression on the cell surface in some, but not all, cell types. Cycloheximide acts by preventing the peptidyl transferase activity of the 60S ribosomal subunit and is widely used as a general purpose inhibitor of eukaryotic protein

a

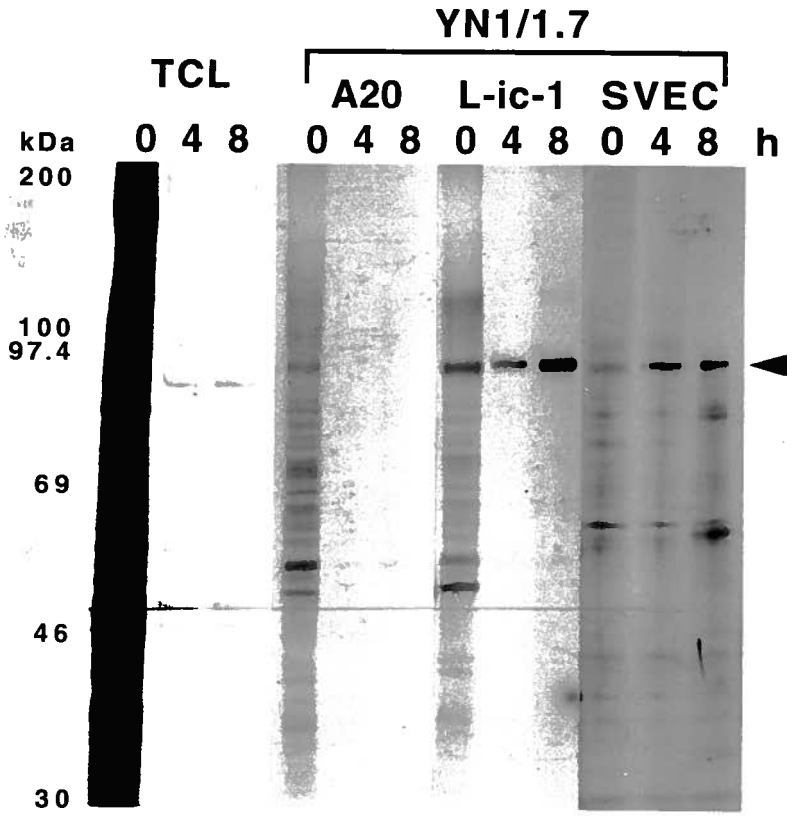
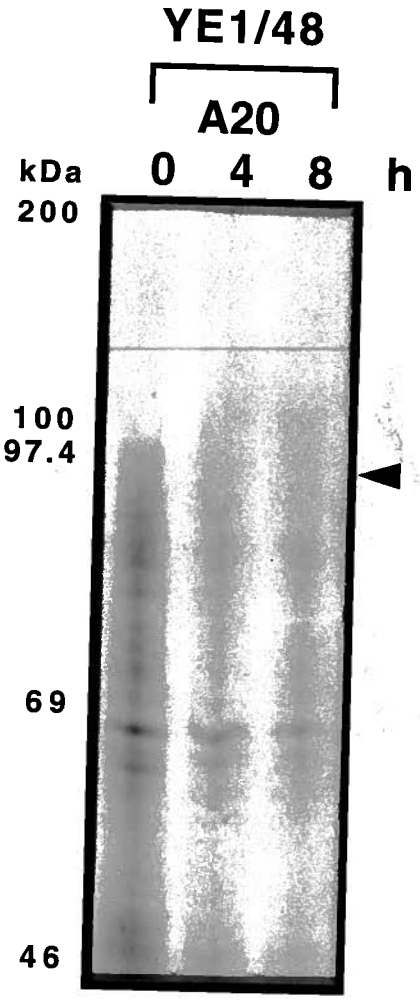


Fig. 21 Effect of cycloheximide and puromycin on *de novo* ICAM-1 protein synthesis. **a.** Cells were treated with cycloheximide for the increasing periods of time. *De novo* ICAM-1 proteins that had incorporated [^{35}S]-methionine were immunoprecipitated from A20, L-ic-1, and SVEC4-10 cells with anti-ICAM-1 mAb and subjected to SDS-PAGE analysis. TCL represents the total cell lysate of L-ic-1. TCLs of A20 and SVEC4-10 gave similar results. The solid arrow marks ICAM-1 proteins (approximately 90 kDa). **b.** A20 cells were treated with cycloheximide for the increasing periods of time as in *a*, but immunoprecipitated with a negative control mAb, YE1/48, and subjected to SDS-PAGE analysis. **c.** SVEC4-10 cells treated with puromycin for the increasing periods of time and immunoprecipitated with anti-ICAM-1 mAb and subjected to SDS-PAGE analysis.. **d.** TCL of L-ic-1 treated with cycloheximide (CHX) alone, in combination with H-7 (H-7 + CHX) and okadaic acid (OkAc + CHX), H-7 alone (H-7), and okadaic acid alone (OkAc).

b



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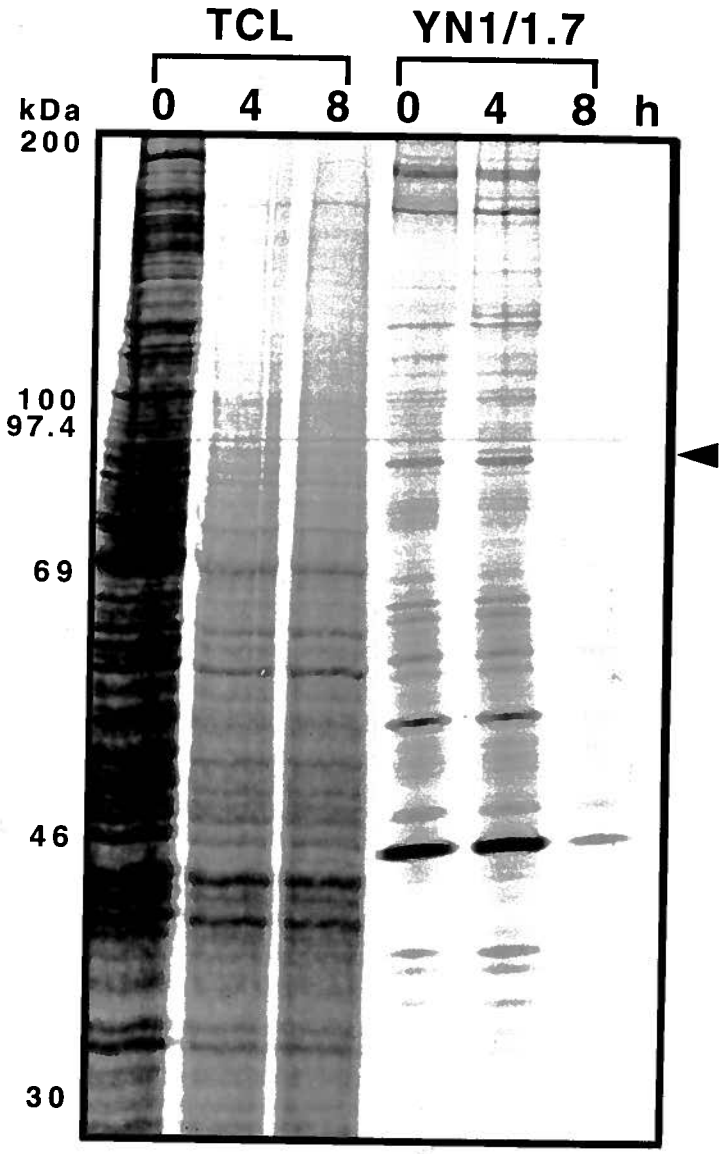

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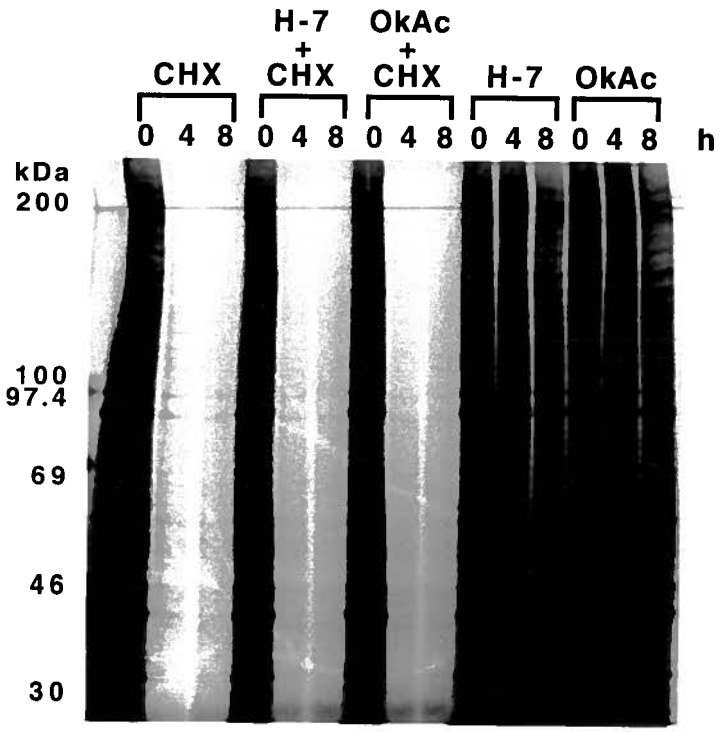
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synthesis. Although its ability to stabilize and superinduce labile mRNAs is well known, the precise mechanisms have yet to be elucidated. Two models have been proposed to explain the effect of cycloheximide (for review, see Sachs, 1993). One states that the degradation of mRNA requires its translation. Therefore, inhibition of translation by cycloheximide results in the stabilization of mRNA. The second model states that a unique class of proteins that have a rapid turnover are required for the degradation of certain highly labile mRNAs. Within this model, cycloheximide might be envisaged to inhibit the synthesis of these proteins, resulting in a quick down-regulation of mRNA degradation. Our finding that cycloheximide stabilizes ICAM-1 mRNA without inhibiting the synthesis of ICAM-1 protein is incompatible with the first model and favours the second.

It was quite unexpected that the synthesis of ICAM-1 determined by [^{35}S]-methionine labeling and immunoprecipitation would actually be increased in cycloheximide-treated cells for two of the lines tested (transfected L cells and endothelial cells) while the overall protein synthesis in the same cells was profoundly inhibited. The increase observed in the synthesis of ICAM-1 and its expression level on the cell surface can be explained by the increased level of ICAM-1 mRNA observed subsequent to cycloheximide treatment. However, how ICAM-1 protein synthesis in some cells escapes the general cycloheximide-mediated inhibition of protein synthesis remains unclear.

Our results also suggest that ser/thr phosphorylation of unknown proteins can regulate the effect of cycloheximide on ICAM-1 mRNA stability. The ser/thr kinase inhibitors, H-7 and staurosporine, completely inhibited the accumulation and stabilization of ICAM-1 mRNA by cycloheximide. On the other hand, the ser/thr phosphatase inhibitor, okadaic acid, alone increased the level of ICAM-1 transcripts by stabilizing them and also augmented the cycloheximide effect. In contrast, other inhibitors including the protein tyrosine kinase inhibitors, genistein and tyrphostein, and the tyrosine phosphatase inhibitor, vanadate, had no measurable effect on ICAM-1 mRNA induction by cycloheximide. Therefore, ser/thr phosphorylation seems to be an important regulatory step in the mechanism by which

cycloheximide controls ICAM-1 mRNA stability. Based on the second model, which proposes the presence of highly labile proteins involved in the degradation of mRNA, these findings can be interpreted as follows. The turnover rate of the putative labile proteins may be regulated by their phosphorylation at the ser/thr residues. This model postulates that in the absence of any inhibitors, the putative labile proteins are synthesized and are in equilibrium between phosphorylated and dephosphorylated forms due to ser/thr kinases and phosphatases. Cycloheximide reduces the level of the labile proteins as it inhibits their synthesis. In the presence of staurosporine or H7, which inhibits kinases, the labile proteins are dephosphorylated by phosphatases and become stable. Therefore, even though cycloheximide inhibits the *de novo* synthesis of the labile proteins, the pre-existing labile proteins that have become stable continue to degrade ICAM-1 mRNA. On the other hand, okadaic acid inhibits phosphatases and induces phosphorylation of the proteins, promoting their degradation. Therefore, the level of the labile proteins declines and ICAM-1 mRNA becomes stable. When both cycloheximide and okadaic acid are added, the level of the labile proteins further declines as their synthesis is inhibited and their degradation is enhanced, resulting in even further stabilization of ICAM-1 mRNA. It is unkown whether the putative labile protein(s) itself degrades mRNA or it recognizes specific motifs in mRNA, thereby acting as a signal recruiting RNases to bind and digest mRNA.

Inflammatory mediators including IFN-γ, TNF-α, and IL-1, as well as active phorbol esters such as PMA, have been shown to dramatically increase the expression of ICAM-1 (Dustin *et al.*, 1986; Rothlein *et al.*, 1988). The enhanced expression of ICAM-1 on venule endothelium is an important step in establishing an inflammatory response. This facilitates the adherence and subsequent diapedesis of leukocytes bearing LFA-1 or Mac-1 into the sites of inflammation (Wawryk *et al.*, 1989; Smith *et al.*, 1989), as well as permitting appropriate interaction of lymphocytes with cells expressing targeted antigens (Dougherty *et al.*, 1988; Wawryk *et al.*, 1989; Springer, 1990). The induction of ICAM-1 expression by certain proinflammatory mediators has been attributed to the stabilization of an otherwise labile

ICAM-1 mRNA. Further elucidation of the mechanisms underlying the regulation of ICAM-1 mRNA turnover will not only allow us to learn more about the posttranscriptional regulation in general but may also help us to know more about the inflammatory response, and may provide new targets for clinically modulating the course of inflammatory diseases.

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V

SUMMARY AND GENERAL DISCUSSION

ICAM-1 is one of several recently described cell adhesion molecules that belongs to the immunoglobulin superfamily and serves as a ligand for the β_2 integrins LFA-1 (Rothlein *et al.*, 1986; Marlin and Springer, 1987; Makgoba *et al.*, 1989) and Mac-1 (Diamond *et al.*, 1991). Adhesion of ICAM-1 to these leukocyte integrins plays an essential role in a variety of immune reactions including T cell-mediated killing, natural lytic events, T-helper and B-lymphocyte responses, homotypic aggregation, antibody-dependent cytotoxicity mediated by monocytes and granulocytes, and leukocyte trafficking processes such as adherence of leukocytes to vascular endothelium and epidermal cells (Springer, 1990; Wawryk *et al.*, 1989).

In contrast to LFA-1, which is expressed only on leukocytes in a constitutive manner, ICAM-1 is an inducible cell surface glycoprotein expressed at a low level on a wide variety of cells, including leukocytes, vascular endothelium, fibroblasts, follicular dendritic cells, and certain epithelial cells (Dustin *et al.*, 1986; Dustin and Springer 1991; Rothlein *et al.*, 1988; Pober *et al.*, 1986; Carlos and Harlan, 1994). ICAM-1 expression is dramatically increased at sites of inflammation, (Springer, 1990; Dustin *et al.*, 1986; Dustin and Springer 1991; Rothlein *et al.*, 1988; Pober *et al.*, 1986; Carlos and Harlan, 1994) providing important means of regulating cell-cell interactions and thereby presumably inflammatory responses. The upregulated expression of ICAM-1 on venule endothelium is thought to facilitate the adhesion and subsequent trans-endothelial migration of leukocytes bearing LFA-1 or Mac-1 into inflammatory tissues, as well as permitting appropriate interaction of lymphocytes with cells expressing targeted antigens (Springer, 1990; Wawryk *et al.*, 1989; Carlos and Harlan, 1994; Dustin and Springer 1991). Various proinflammatory mediators including IL-1, TNF-α, LPS, and IFN-γ, as well as active phorbol esters, have been found to increase ICAM-1 expression on

many cell types (Dustin *et al.*, 1986; Rothlein *et al.*, 1988; Carlos and Harlan, 1994) and are thought to be responsible for the induction of ICAM-1 expression at inflammatory sites *in vivo*. The function and indeed the importance of leukocyte adhesion in the generation and maintenance of inflammation were elucidated in numerous animal experimental systems where monoclonal antibodies to ICAM-1 were used as antagonists and found to inhibit multiple events associated with inflammation (Carlos and Harlan, 1994).

The importance of leukocyte integrins and their counter-receptors is further illustrated in congenital LAD I syndrome (Springer, 1990; Carlos and Harlan, 1994; Dustin and Springer 1991) in which the leukocyte integrins are deficient due to genetic mutations in the common β_2 subunit CD18. As described in chapter I, these patients are immunodeficient and thus have seriously compromised ability to fight frequent infections. Furthermore, ICAM-1-deficient mice have been generated recently and showed reduced inflammatory response (Sligh *et al.*, 1993; Xu *et al.*, 1994). Moreover, the ICAM-1 protein has been involved in various pathological processes such as serving as a receptor for the major serotype of rhinoviruses (Greve *et al.*, 1989; Staunton *et al.*, 1989a; Springer, 1990) and being subverted as a sequestration antigen for *P. falciparum*-infected RBCs (Berendt *et al.*, 1989), and its expression is thought to correlate positively with the metastatic potential of malignant melanoma (Johnson *et al.*, 1989).

Thus, the regulated expression of ICAM-1 by various tissues and cell types appears to play a vital role in numerous physiologic and pathologic processes of the immune system. Development of pharmacologic strategies to achieve the modulation of ICAM-1 expression during inflammation might therefore be of medical interest, but requires a knowledge of the intracellular regulatory elements and signaling pathways that underlie the inducible expression of ICAM-1 by proinflammatory cytokines. The results in this thesis shed a new light into the regulation of ICAM-1 gene expression at a posttranscriptional level by two inflammatory mediators, IFN-γ and PMA, and provide evidence that a ser/thr phosphorylation pathway is involved in cycloheximide-induced ICAM-1 message stabilization.

5.1 ICAM-1 REGULATION BY IFN-γ AND PMA

5.1.1 Transcriptional Regulation Does Not Tell the Whole Story

The proinflammatory cytokine IFN-γ as well as the PKC activator PMA markedly increase the expression of ICAM-1 on various cells of both hematopoietic and non-hematopoietic origins (Dustin *et al.*, 1986; Rothlein *et al.*, 1988). Because IFN-γ enhances the transcription of many IFN-γ-responsive genes including the class II major histocompatibility complex (Moses *et al.*, 1992), its effect on ICAM-1 has also been thought to be similar. Indeed, within the 5'-flanking region of ICAM-1 gene are contained potential IFN-γ-responsive elements, glucocorticoid receptor-binding sites, NF-κB consensus elements, and AP-1/TRE, AP-2 and AP-3-like sites (Degitz *et al.*, 1991; Voraberger *et al.*, 1991). However, these potential elements could only mount a twofold induction by IFN-γ using a reporter assay (Voraberger *et al.*, 1991), suggesting that ICAM-1 gene regulation by IFN-γ may also involve posttranscriptional mechanisms. Similarly, PMA had little effect on transcription of ICAM-1 gene in HUVECs, as measured by the nuclear run-on assay (Wertheimer *et al.*, 1992). In contrast, Voraberger *et al.* (1991) reported that PMA stimulated the expression of a luciferase reporter gene linked to the 5'-flanking region of the ICAM-1 gene containing three copies of TRE in transiently transfected A549 cells. Interestingly, similar results were obtained in HUVECs transfected with the same pBHluc1.3 construct of Voraberger *et al.*, suggesting that although PMA can activate exogenous ICAM-1 enhancer/promoter elements in HUVECs, the same elements in the endogenous gene remain functionally silent (Wertheimer *et al.*, 1992).

5.1.2 Novel Posttranscriptional Regulation of ICAM-1 Gene

There is growing evidence that mRNA turnover plays an important role in regulating gene expression. The steady-state levels of many mRNAs do not necessarily reflect their

transcriptional rates, suggesting that cellular metabolism is influenced by the stability of individual mRNAs and by the ability of the cells to regulate mRNA turnover. In fact, the rapid turnover of an mRNA ensures that it is maintained at relatively low steady-state levels and that changes in the rate of degradation can affect its steady-state level over a short period of time. This type of regulation allows transient alterations in the expression of some cytokines, transcription factors, and proto-oncogenes such as *c-fos*, *c-myc*, and *c-myb* in response to growth factors, phorbol esters, antigen stimulation, or inflammation (Sachs, 1993). This class of short-lived mRNAs all share a common AUUUA sequence motif in their 3'UTRs, which serves as one signal targeting the mRNAs for rapid turnover (Schuler and Cole, 1988; Shaw and Kamen, 1986). The 3'UTRs of both human and murine ICAM-1 mRNAs also contain several AUUUA sequences (Horley *et al.*, 1989; Staunton *et al.*, 1988), and their roles with respect to the inflammatory stimuli are discussed below.

Our recent studies (Ohh *et al.*, 1994) have shown that the treatment of the murine monocytic cell line P388D1 with IFN-γ or PMA induced a rapid increase in the level of endogenous ICAM-1 mRNA. The peak message accumulation (5-8x basal level) was achieved within 2 h after the addition of IFN-γ and around 6 h after the addition of PMA. Analysis of ICAM-1 transcript stability in cells treated with actinomycin D to block new RNA synthesis showed that ICAM-1 mRNA degraded at a substantially slower rate when the cells had been pretreated with IFN-γ or PMA. The half-life of ICAM-1 mRNA from untreated cells was about 50 min, whereas degradation of the message in PMA- or IFN-γ-treated cells was negligible over the 2 h duration of the experiment. Furthermore, the translation inhibitor cycloheximide, which can stabilize transiently expressed messages, increased the ICAM-1 mRNA content of unstimulated cells. These observations led us to conclude that the up-regulation of ICAM-1 mRNA level by IFN-γ and PMA was due, at least in part, to the stabilization of the message.

Although an effect of IFN-γ on ICAM-1 mRNA stability had not been previously recognized, it should be noted that message stabilization by PMA is not unique to ICAM-1

gene expression. In fact, PMA treatment has been shown to stabilize transcripts for transforming growth factor-β1, IL-1, IL-3, and *c-fms* (Wager and Assoian, 1990; Yomoto *et al.*, 1989; Wodnar-Filipowicz and Moroni, 1990; Weber *et al.*, 1989). Furthermore, PMA treatment of mononuclear cells resulted in an increased stability of labile messages, such as *c-fos* mRNA, which was associated with enhanced adenosine-uridine binding factor that specifically bind to the AUUUA sequence (Gillis and Malter, 1991). Conversely however, the stabilization of lymphokine (IL-2, TNF-α, and GM-CSF) mRNAs induced by costimulation with PMA correlated inversely with the binding of a sequence-specific cytoplasmic factor that binds specifically to AUUUA multimers present in the 3'UTR of lymphokine mRNAs (Bohjanen *et al.*, 1991).

To investigate whether the AUUUA motifs in the 3'UTR of ICAM-1 mRNA serve as a destabilizing signal and to delineate the elements responsive to IFN-γ and PMA, a range of deletion mutants of murine ICAM-1 cDNA were generated (Figure 22) and stably transfected into the murine fibroblast Ltk^- cells, which do not express endogenous ICAM-1 (Ohh and Takei, 1994). As summarized in Fig. 22, the induction of ICAM-1 mRNA in the transfectants by treatments with IFN-γ revealed that truncation of the region of ICAM-1 mRNA encoding the cytoplasmic domain made it unresponsive to IFN-γ whereas all other regions were dispensable. This suggests that the region of 87 nt within ICAM-1 mRNA encoding the cytoplasmic domain contained IFN-γ-responsive element(s) (Ohh and Takei, 1994).

In contrast, PMA-induced accumulation of ICAM-1 mRNA required the 3'UTR containing multiple AUUUA pentanucleotides (Ohh and Takei, 1994). To further elucidate the role of these regions in mRNA destabilization and responsiveness to IFN-γ and PMA, ICAM-2 mRNA that is stable and not responsive to IFN-γ or PMA was used as a reporter gene. The putative IFN-γ-responsive region of ICAM-1 mRNA encoding its cytoplasmic domain rendered it unstable and responsive to IFN-γ but not PMA. Conversely, the 3'UTR of ICAM-1 ligated with ICAM-2 mRNA also made it labile and responsive to PMA but not IFN-γ. Half-life analysis showed that the increased levels of these chimeric mRNAs induced by IFN-γ and

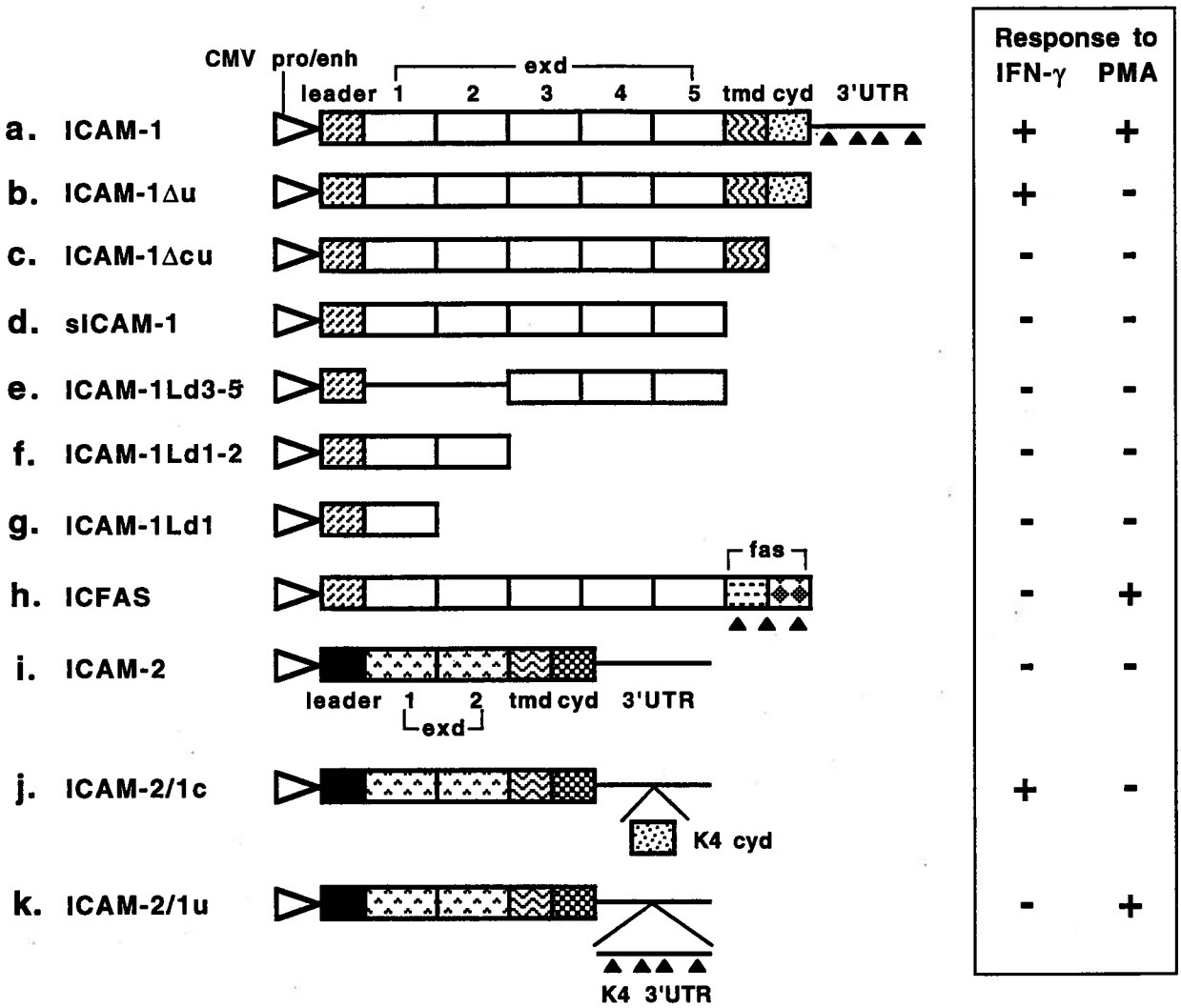

Fig. 22 Summary of the responses of various ICAM-1 mRNA deletion mutants and ICAM-2/1 chimeric mRNAs to IFN-γ and PMA. + represents induction and stabilization of message and – represents a negligible response. **a.** ICAM-1; full-length ICAM-1 expression vector. **b.** ICAM-1Δu; ICAM-1 without the 3'UTR. **c.** ICAM-1Δcu; ICAM-1 without the cytoplasmic domain and the 3'UTR. **d.** sICAM-1; soluble ICAM-1 without the 3'UTR, cytoplasmic and transmembrane domains. **e.** ICAM-1Ld3-5; soluble ICAM-1 without domains 1 and 2. **f.** ICAM-1Ld1-2; soluble ICAM-1 without domains 3 to 5. **g.** ICAM-1Ld1; soluble ICAM-1 without domains 2 to 5. **h.** ICAM-2; full-length ICAM-2 expression vector. **i.** ICAM-2/1c; cytoplasmic domain of ICAM-1 incorporated into ICAM-2 3'UTR. **j.** ICAM-2/1u; 3'UTR of ICAM-1 incorporated into ICAM-2 3'UTR. Open triangle represents CMV promoter/enhancer; Dashed square, ICAM-1 leader sequence; Open rectangles, ICAM-1 extracellular domains (exd); Vertical wavy square, ICAM-1 transmembrane domain (tmd); Dotted square, ICAM-1 cytoplasmic domain (cyd); Solid triangles, AUUUA pentamers within ICAM-1 3'UTR; Solid square, ICAM-2 leader sequence; Spotted rectangles, ICAM-2 extracellular domains; Horizontal wavy square, ICAM-2 transmembrane domain; Checkered square, ICAM-2 cytoplasmic domain.

PMA were due, at least in part, to a prolongation of their turnover rate (Ohh and Takei, 1994). Hence, it seems likely that ICAM-1 mRNA contains at least two distinct elements which are responsive to specific cell activation signals (Fig. 23).

Although the 3'UTR containing the putative AUUUA destabilzing motifs seem to serve as a mechanism of PMA-induced ICAM-1 transcript stabilization, it may yet have another function. ICAM-1 mRNA missing the 3'UTR was observed having a higher basal level than the mRNA with the 3'UTR, and this was demonstrated to be due to the increased stability (Ohh *et al.*, 1994). In support, Bevilacqua *et al.* (1987) have noted that the apparent instability of the ELAM-1 mRNA in response to TNF-α stimulation may be due to the presence of seven copies of the AUUUA motif within its 3'UTR. In comparison, another LFA-1 ligand ICAM-2, which is constitutively expressed on vascular endothelium (Staunton *et al.*, 1989b; Xu *et al.*, 1992; de Fougerolles *et al.*, 1991), has a much higher basal level of expression than ICAM-1, and is not further induced by proinflammatory cytokines (Staunton *et al.*, 1989b; de Fougerolles *et al.*, 1991). Similarly, the recently cloned ICAM-3 appears to be the primary ligand for LFA-1 on resting lymphocytes, and like ICAM-2, it is expressed in a constitutive manner (Fawcett *et al.*, 1992; Vazeux *et al.*, 1992). Interestingly, unlike ICAM-1, both ICAM-2 and -3 mRNAs do not possess AUUUA destabilizing sequences in their 3'UTRs. Hence, these putative destabilizing sequences within the 3'UTR may be one of several mechanisms maintaining the expression of ICAM-1 at low or undetectable levels in the absence of an inflammatory response *in vivo*. Such down-regulation of ICAM-1 would play a crucial role in minimizing unwanted lymphocyte antigen-specific responses and leukocyte trafficking, as well as to promote the protraction of an inflammatory response.

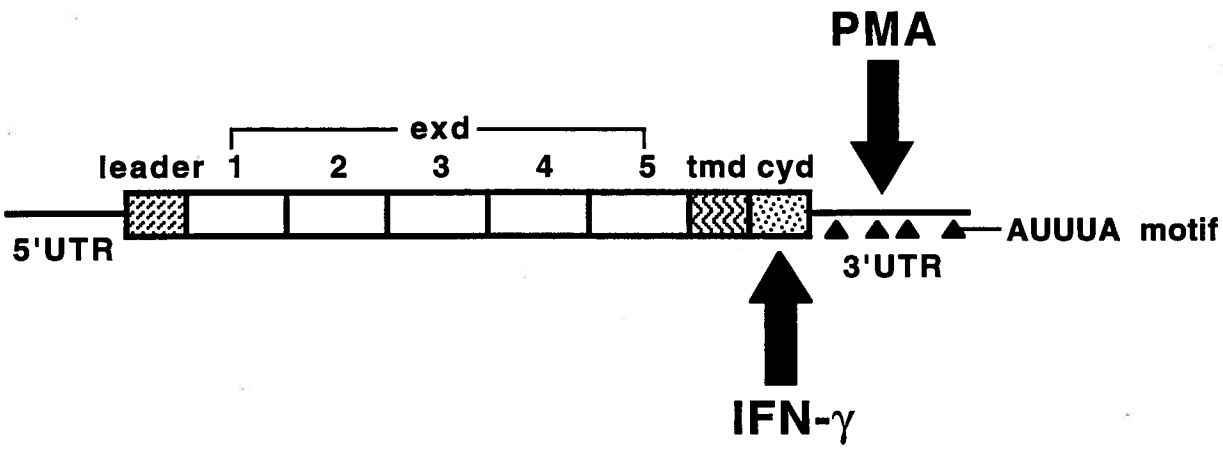

Fig. 23 Schematic diagram of ICAM-1 gene showing the location of the putative IFN-γ- and PMA-responsive elements responsible for the stabilization of ICAM-1 message. Symbols are the same as those used in *Fig. 22*. Shaded arrow indicates the region of the corresponding inflammatory mediator-responsive element(s).

5.2 ROLE OF SERINE/THREONINE PHOSPHORYLATION IN ICAM-1 mRNA STABILIZATION

Cycloheximide is an eukaryotic protein synthesis inhibitor that superinduces the expression of many genes by preventing the degradation of otherwise labile mRNAs. For this reason, cycloheximide has been widely used to aid in the characterization of the mechanisms responsible for message stabilization (Jackson, 1993; Sachs, 1993). From these studies, it has been suggested that mRNAs are degraded by a class of highly labile proteins (Jackson, 1993; Sachs, 1993). However, upon cycloheximide treatment, these proteins are no longer synthesized but continue to degrade, resulting in message stabilization. We examined the effect of cycloheximide on the murine ICAM-1 gene expression in several cell lines including A20 (B cell lymphoma), T28 (T cell hybridoma), P388D1 (monocytic cell), SVEC4-10 (lymphoid endothelial cell), and ICAM-1-transfected murine fibroblast Ltk^- cells (Ohh and Takei, 1995). Cycloheximide was indeed able to dramatically increase the accumulation of ICAM-1 mRNA in all the cell lines examined except T28, and this seemed to be due to the stabilization of the ICAM-1 mRNA as indicated by the half-life analysis. Interestingly, the effect of cycloheximide on ICAM-1 mRNA was markedly suppressed by ser/thr kinase inhibitors, H-7 and staurosporine, whereas the ser/thr phosphatase inhibitor, okadaic acid, augmented the cycloheximide effect. In contrast, inhibitors of protein tyrosine kinases and phosphatases had no effect. Therefore, ser/thr phosphorylation seems to be an important regulatory mechanism for the effect of cycloheximide on ICAM-1 mRNA stability. Based on the model which proposes the presence of highly labile proteins involved in the degradation of mRNA, these findings can be interpreted as follows (Fig. 24): The turnover rate of the putative labile proteins may be regulated by their phosphorylation at the ser/thr residues. This model postulates that in the absence of any inhibitors, the putative labile proteins are synthesized and are in equilibrium between phosphorylated and dephosphorylated forms due to ser/thr kinases and phosphatases. Cycloheximide reduces the level of the labile proteins as it

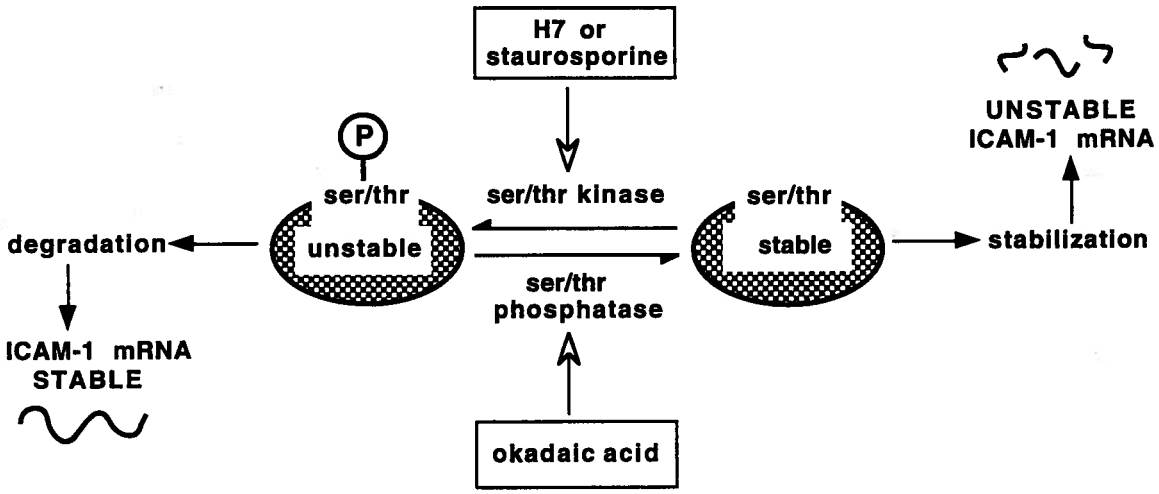

Fig. 24 Schematic diagram of the serine/threonine phosphorylation pathway involved in the stabilization of ICAM-1 mRNA by cycloheximide. Checkered circle represents the highly labile protein that degrades ICAM-1 mRNA. Circled P represents the phosphorylated serine/threonine (ser/thr) residue(s) on the labile protein.

inhibits their synthesis. In the presence of staurosporine or H7, which inhibits kinases, the labile proteins are dephosphorylated by phosphatases and become stable. Therefore, even though cycloheximide inhibits the *de novo* synthesis of the labile proteins, the pre-existing labile proteins that have become stable continue to degrade ICAM-1 mRNA. On the other hand, okadaic acid inhibits phosphatases and induces phosphorylation of the proteins, promoting their degradation. Therefore, the level of the labile proteins declines and ICAM-1 mRNA becomes stable. When both cycloheximide and okadaic acid are added, the level of the labile proteins further declines as their synthesis is inhibited and their degradation is enhanced, resulting in even further stabilization of ICAM-1 mRNA. It is unkown whether the putative labile protein(s) itself degrades mRNA or it recognizes specific motifs in mRNA, thereby acting as a signal recruiting RNases to bind and digest mRNA.

5.3 CONCLUDING REMARKS

Although LFA-1 and its ligands ICAM-2 and -3 are constitutively expressed on restricted cell types, ICAM-1 is an inducible cell surface glycoprotein expressed on a wide variety of cells. The induction of ICAM-1 expression at sites of inflammation is an important means of regulating ICAM-1-dependent adhesion and thereby inflammatory responses. If ICAM-1 can be down-regulated and/or cytokine effects on the induction of ICAM-1 inhibited, it would be possible that the inflammatory response can be dampened. Inasmuch as ICAM-1 is expressed at a low basal level on leukocytes, vascular endothelium, fibroblasts, and certain epithelial cells, its induction by proinflammatory cytokines makes it an attractive target for regulation. Moreover, finding ways of down-regulating an already high expression of ICAM-1 will undoubtedly be important in inhibiting an already protracted inflammatory response. Toward this end, we have described a novel mechanism of regulating ICAM-1 gene expression by two inflammatory mediators, IFN-γ and PMA, and found that both induce the message

accumulation by stabilizing an otherwise labile mRNA through two distinct destabilizing elements; the 87 nt region encoding the cytoplasmic domain and the 3'UTR containing multiple AUUUA motifs were responsive to IFN-γ and PMA, respectively. Furthermore, ser/thr phosphorylation of unidentified protein(s) seems to play a crucial role in the stabilization of ICAM-1 message by cycloheximide. Further characterization of the complex regulatory mechanisms involved in ICAM-1 expression should enhance our understanding of the inflammatory process and may thereby provide new strategies for modulating the course of inflammatory diseases.

It should be noted that although we have identified destabilizing regions both in the 3'UTR and the ORF of ICAM-1 mRNA, other mRNAs such as *c-myc* and *c-fos* also have destabilizing regions within their 3'UTR and ORF (Shyu *et al.*, 1989; Bernstein *et al.*, 1992). Moreover, it has been reported that cycloheximide can have stabilizing effect on labile mRNAs, such as *c-jun*, even at concentrations that do not inhibit translation (Rao and Mufson, 1993). Hence, ICAM-1 translational escape from cycloheximide may not be a unique phenomenon.

5.4 FUTURE DIRECTIONS

Delineation of the destabilizing regions responsive to various proinflammatory mediators may be an important step in understanding the regulation of ICAM-1 gene expression. The identification of such destabilizing sequences in ICAM-1 mRNA will lead to isolation of proteins that bind to these elements. Characterization of proteins that bind to the specific sequences will undoubtedly shed new light into the mechanisms that control mRNA stability. Such discoveries may help to discern between a common pathway or complex individual pathways involved in the posttranscriptional regulation of gene expression, which is thought to be particularly important for the regulation of highly labile mRNAs including ICAM-1 mRNA.

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