Journal of Cellular Biochemistry 59:202–213 (1995)

Regulation of ICAM-1 mRNA Stability by Cycloheximide: Role of Serine/Threonine Phosphorylation and Protein Synthesis

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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. We examined the effect of cycloheximide on the murine intercellular adhesion molecule-1 (ICAM-1; CD54) 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 L cells. 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. To determine whether this effect is dependent on the 3'UTR containing the AUUUA sequences, L cells were transfected with either the full-length ICAM-1 cDNA or a truncated form lacking the AUUUA sequences in the 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 transfected 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 was 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 this effect.

Key words: cycloheximide, ICAM-1, mRNA stability, serine, threonine phosphorylation, translation

Intercellular adhesion molecule-1 (ICAM-1; CD54) is an inducible cell surface glycoprotein expressed in various lineages of hematopoietic and non-hematopoietic origin including leukocytes, vascular endothelium, fibroblasts, and certain epithelial cells [Dustin et al., 1986], and serves as a ligand for lymphocyte function-associated antigen-1 (LFA-1; CD11a/CD18) [Rothlein et al., 1986; Marlin and Springer, 1987; Makgoba et al., 1989] and Mac-1 (CD11b/CD18) [Diamond et al., 1991]. Adhesion of ICAM-1 to these leukocyte integrins plays an essential role in a variety of immune reactions including lymphocyte antigen-specific responses and leukocyte trafficking [for review, see Springer, 1990]. Moreover, this versatile molecule is exploited as a receptor for the major serotype of rhinoviruses [Greve et al., 1989; Staunton et al., 1989] and for *Plasmodium falciparum*-infected erythrocytes [Berendt et al., 1989], and its expression is thought to correlate positively with the metastatic potential of malignant melanoma [Johnson et al., 1989].

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. Various inflammatory mediators, such as interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-1, and lipopolysaccharide (LPS) have been found to upregulate

Received January 19, 1995; revision accepted February 16, 1995.

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ICAM-1 expression on the surface of many cell types [Dustin et al., 1986; Rothlein et al., 1988]. The enhanced expression of ICAM-1 on venule endothelium facilitates the adhesion and subsequent trans-endothelial migration of leukocytes bearing LFA-1 or Mac-1 into inflammatory tissues [for reviews 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].

Despite the obvious importance of inducible ICAM-1 expression in numerous immunologic responses, very little is known about the intracellular mechanisms controlling its expression. There is, however, growing evidence that mRNA turnover plays an important role in regulating gene expression. 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′ untranslated regions (UTRs), which serves as one signal targeting the mRNAs for rapid turnover [Shaw and Kamen, 1986; Schuler and Cole, 1988]. 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.

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]. Concordantly, the degradation of the mRNAs for histone, β-tubulin, transferrin receptor, certain proto-oncogenes, and lymphokines is dramatically reduced in the presence of translational inhibitors [for reviews, see Jackson, 1993; 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 remains to be elucidated.

We report here that cycloheximide stabilizes otherwise labile ICAM-1 mRNA but this effect is independent of the 3′UTR containing multiple AUUUA sequences. Inhibitors of serine/threonine (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 independent of the inhibition of its translation.

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 *Pst I* 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 *Xba I* and *Not I*. The sICAM-2, truncated (ICAM-1Δ3) and the full-length ICAM-1 cDNAs were blunt-ligated into *Sma I* 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 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

Cells (1.5×10^6) were pretreated for 15 min at 37°C with various phosphorylation inhibitors (all from Calbiochem), including staurosporine (0.2 μM) H-7 (10 μM), H-89 (0.1 μM), bisindolylmaleimide (0.03 μM), okadaic acid (0.5 μM), genistein (20 μM), tyrphostein (30 μM), and vanadate (50 μM). 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

Cells (1.5×10^6) were treated with various phosphorylation inhibitors in combination with cycloheximide for 2 h at 37°C, and then actinomycin D (10 μg/ml, GIBCO) 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

Total RNA was prepared as described by 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). Fifty microliters 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 centrifuged 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 and transferred onto nylon membranes (Bio-Rad Laboratories, Mississauga, Ontario). 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.

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

Cells (1.5×10^6) cultured in DMEM + 10% FCS were stimulated with cycloheximide (10 μg/ml) 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. 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. Sixty microliters of the supernatant were 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.

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. 1). 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. 2A) 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. 2B). To exclude the possibility that the effects of cycloheximide on ICAM-1 mRNA might be mediated by the expression vector that provided the 3'UTR for ICAM-1Δ3 mRNA, stable ICAM-2 mRNA [Ohh et al., 1994; Ohh and Takei, 1994] was used as a control. Truncated ICAM-2 cDNA (sICAM-2) that lacks its 3'UTR was subcloned into the same expression vector and transfected into L cells. Only a low level induction of sICAM-2 mRNA was achieved by cycloheximide treatment (Fig. 2c), indicating that the 3'UTR supplied by the vector, which is derived from the rabbit globin mRNA, is not responsive to cycloheximide. These results indicate that the 3'UTR containing the AUUUA multimers is not required for the induction of ICAM-1 mRNA by cycloheximide.

Ser/Thr Kinase and Phosphatase Inhibitors and Cycloheximide Effect

Phorbol myristate acetate (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 induction of ICAM-1 mRNA by cycloheximide, ICAM-1-transfected L cells (L-ic-1) were pretreated with various protein kinase and phosphatase inhibitors, prior to the addition of cycloheximide, and their effects on ICAM-1 mRNA induction were determined. Two ser/thr kinase inhibitors, staurosporine and H-7, completely inhibited the induction of ICAM-1 mRNA by cycloheximide (Fig. 3A), whereas the protein tyrosine kinase inhibitor genistein and the protein tyrosine phosphatase inhibitor vanadate had no effect. Interestingly, the ser/thr phosphatase in-

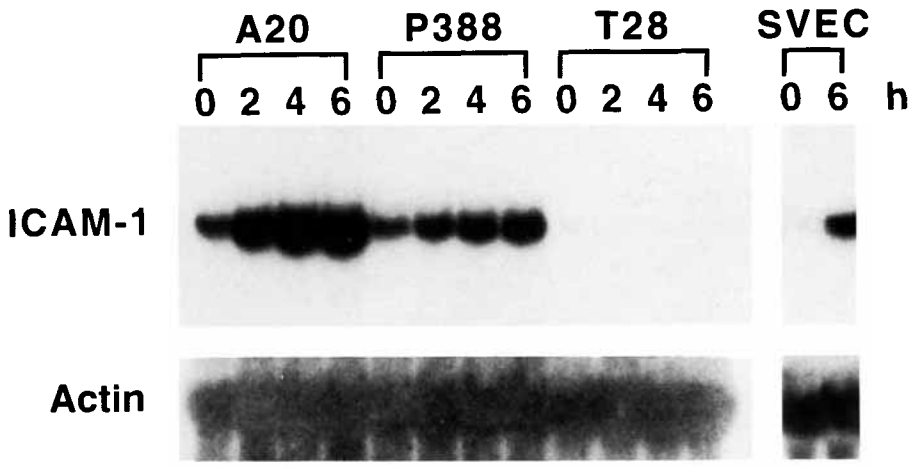

Fig. 1. Effects of cycloheximide on ICAM-1 mRNA expression. A20, P388D1, T28, and SVEC4-10 cells were treated with cycloheximide for the increasing periods of time and the ICAM-1 mRNA levels were detected by Northern blot analysis. The bottom panels represent the signals of the control β-actin. The data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

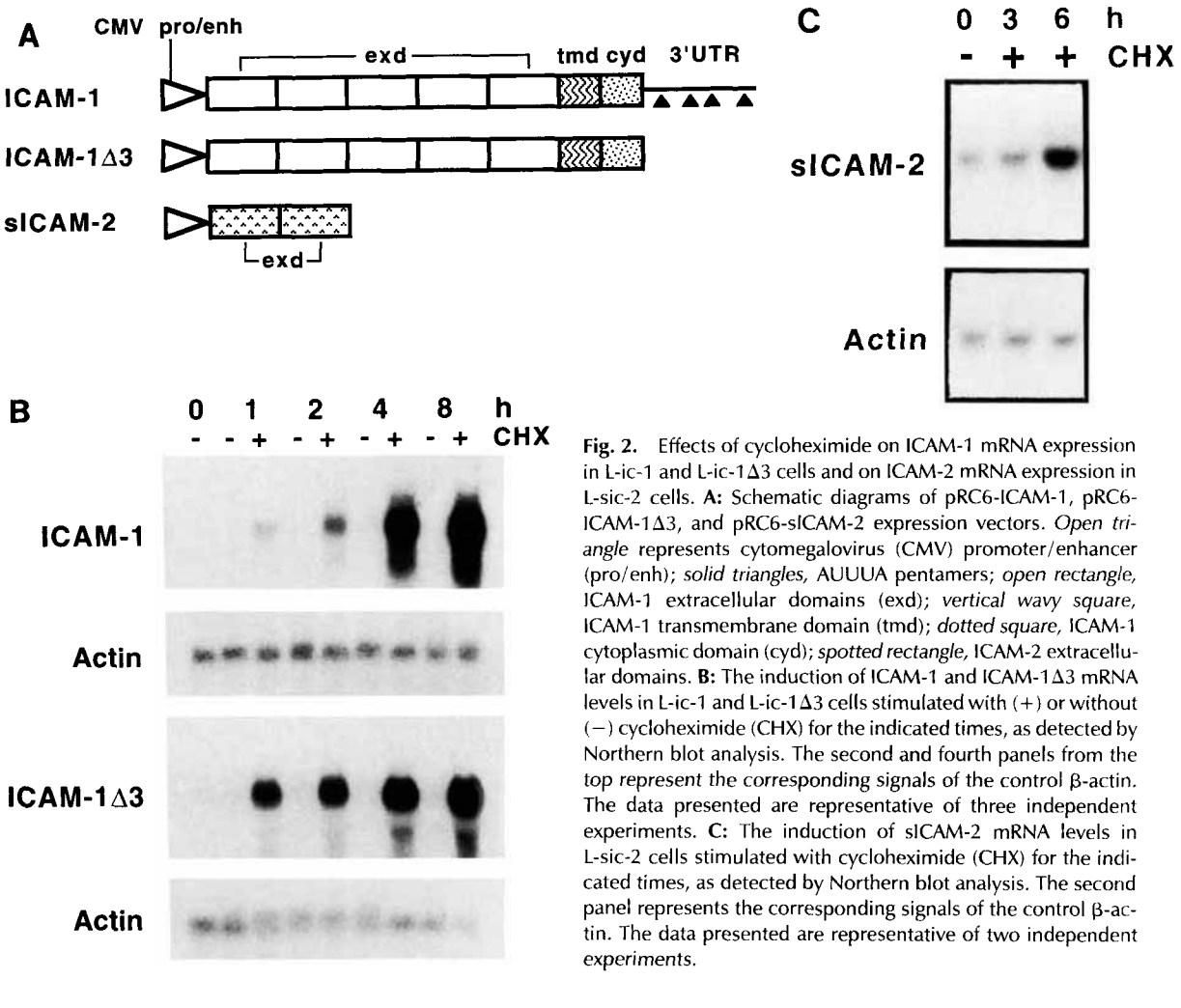

Fig. 2. Effects of cycloheximide on ICAM-1 mRNA expression in L-ic-1 and L-ic-1Δ3 cells and on ICAM-2 mRNA expression in L-sic-2 cells. **A:** Schematic diagrams of pRC6-ICAM-1, pRC6-ICAM-1Δ3, and pRC6-sICAM-2 expression vectors. *Open triangle* represents cytomegalovirus (CMV) promoter/enhancer (pro/enh); *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.

hibitor okadaic acid induced ICAM-1 mRNA in the absence of cycloheximide and also further enhanced the effect of cycloheximide on ICAM-1 mRNA. On the other hand, depletion of protein kinase C by prolonged exposure of the transfected L cells with PMA did not inhibit the induction of ICAM-1 mRNA by cycloheximide, whereas addition of staurosporine to PMA-treated cells completely inhibited the induction of ICAM-1 mRNA (Fig. 3B), suggesting that protein kinase C is not involved in the induction of ICAM-1 by cyclohximide. In agreement with these results, 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. 3C). These results, taken together, suggest that cycloheximide induces the accumulation of ICAM-1 through a ser/thr phosphorylation-dependent pathway.

Half-Life Analysis

Although ICAM-1 mRNA has a short half-life (Fig. 4A,B), treatment with cycloheximide dramatically prolonged the half-life to far greater than 2 h (Fig. 4A). Treatment with the ser/thr kinase inhibitors, staurosporine and H-7, alone had a negligible effect on the turnover rate (Fig. 4A). However, the cycloheximide effect was efficiently inhibited by these ser/thr kinase inhibitors (Fig. 4A). Treatment with the ser/thr phosphatase inhibitor, okadaic acid, alone extended the half-life to far greater than 2 h (Fig. 4B). Tyrosine kinase and phosphatase inhibitors did not affect the prolongation of ICAM-1 mRNA half-life by cycloheximide (data not shown). 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

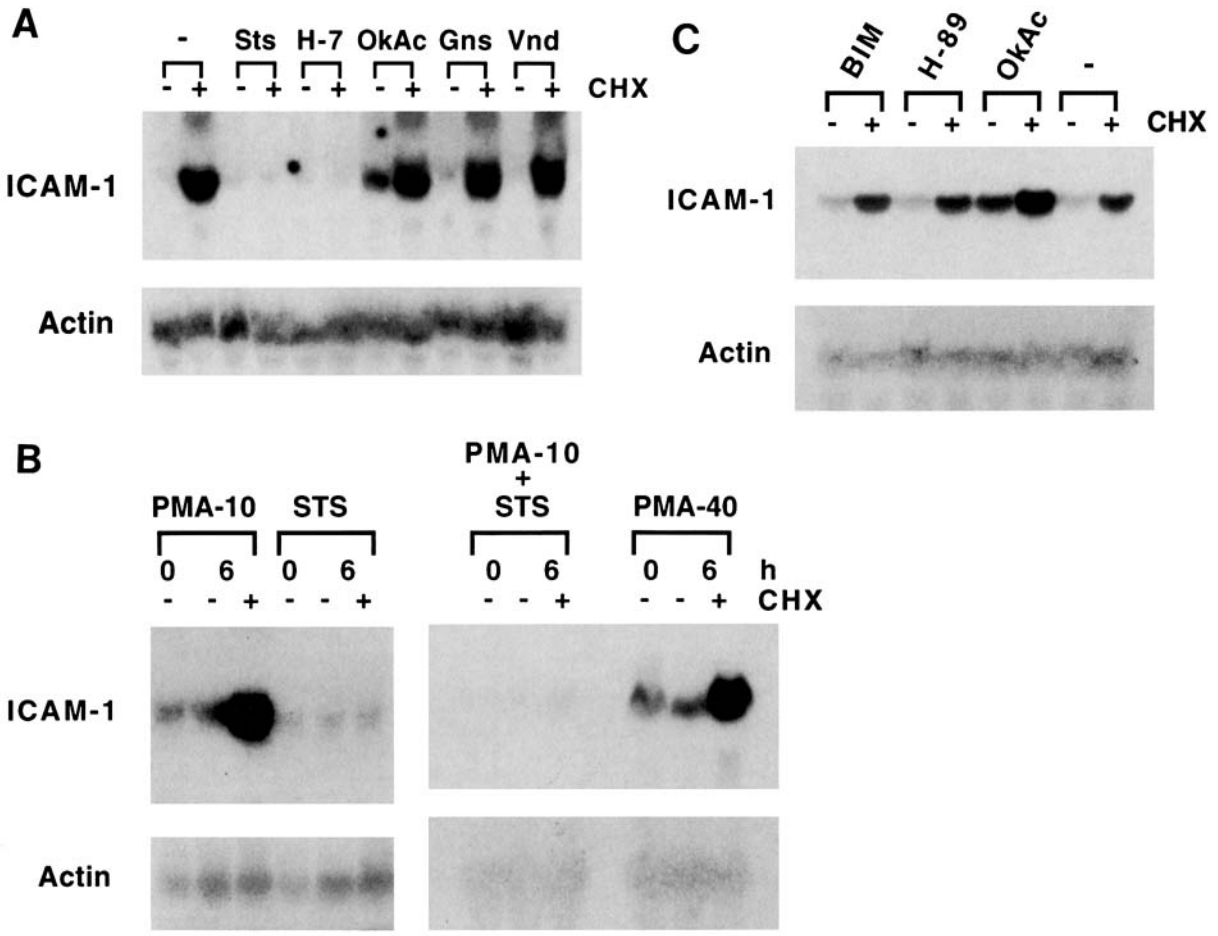

Fig. 3. Effects of phosphorylation inhibitors on ICAM-1 mRNA accumulation by cycloheximide. **A:** Cells were first treated with various phosphorylation inhibitors (Sts, staurosporine; H-7, H-7; OkAc, okadaic acid; Gns, genistein; Vnd, vanadate; −, no phosphorylation inhibitor added) for 15 min and then the cells were treated with (+) or without (−) cycloheximide (CHX) for 6 h. **B:** Cells were treated with PMA (either 10 or 40 ng/ml; PMA-10 and PMA-40, respectively) for 72 h or staurosporinee alone for 15 min (STS) or PMA for 72 h followed by staurosporinee 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. **C:** 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.

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, and as expected from the induction analysis, treatment of sICAM-2 transfected L cells (L-sic-2) with cycloheximide had a negligible effect on the sICAM-2 mRNA turnover rate (Fig. 4C).

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 ICAM-1 transfected L (L-ic-1) cells (Fig. 5B). Untransfected L cells did not express ICAM-1 on the cell surface regardless of cycloheximide treatment (Fig. 5A). The treatment of the transfected L cells with the tyrosine kinase inhibitors (genistein and tyrphostein), tyrosine phosphatase inhibitor (vanadate), general ser/thr kinase inhibitors

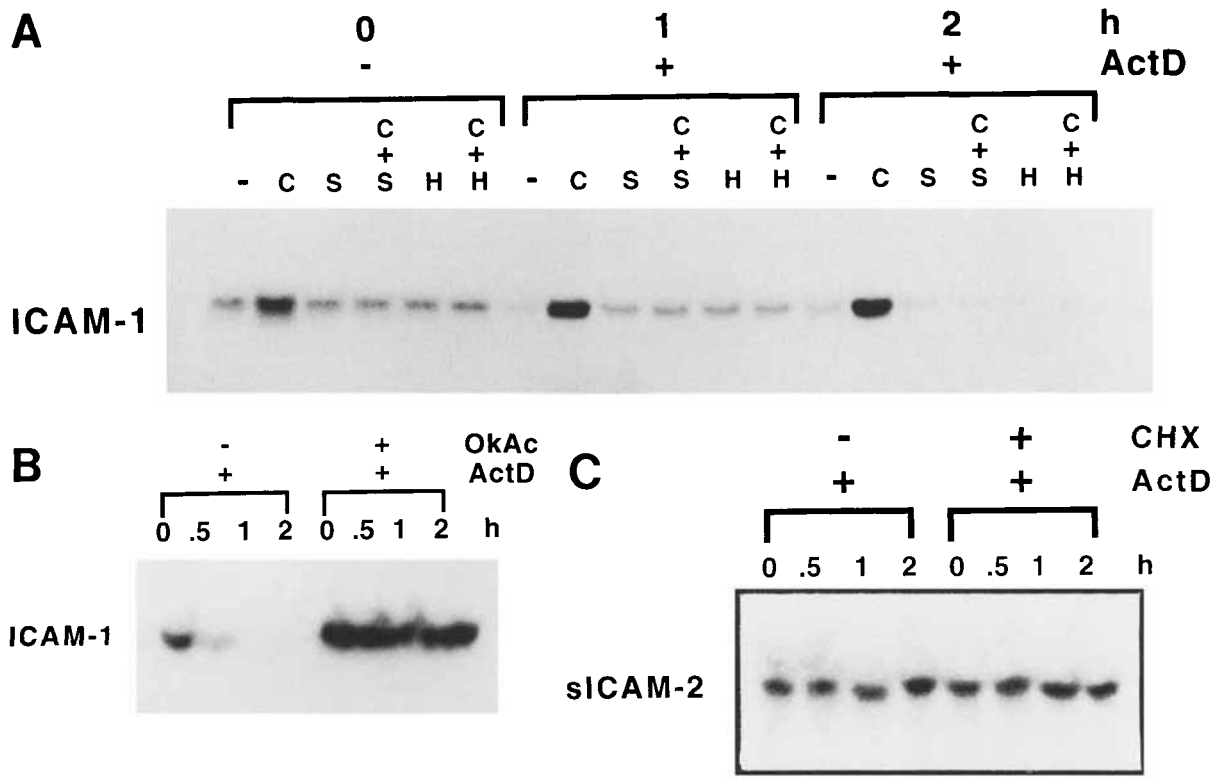

Fig. 4. ICAM-1 and ICAM-2 mRNA half-life analysis. **A:** Cells were treated with phosphorylation inhibitors (S, staurosporine; H, H-7; −, no inhibitor added) for 15 min, followed by stimulation with or without cycloheximide (C) for 2 h. Actinomycin D (ActD) was then added for the indicated times. **B:** Cells were treated with (+) or without (−) okadaic acid (OkAc) for 2 h and then actinomycin D (ActD) 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 (data not shown). 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 (ActD) 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 (data not shown). The data presented are representative of two independent experiments.

(H-7 and staurosporine), and the inhibitors of PKC and PKA (bisindolylmaleimide and H-89, respectively) had no effect on the expression of ICAM-1 (Fig. 5C). Another translational inhibitor, puromycin, alone also had no effect, indicating that the effect of cycloheximide is not general to all protein synthesis inhibitors (Fig. 5C). Okadaic acid alone was able to increase the expression of ICAM-1 (Fig. 5D). The upregulation of cell surface ICAM-1 expression by cycloheximide was markedly reduced by the addition of staurosporine or H-7 (Fig. 5E,F). On the other hand, other inhibitors had no effect on the upregulation of cell surface ICAM-1 expression by cycloheximide (Fig. 5G,H). These results are consistant 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. Nearly identical results were also obtained with the endothelial cell line SVEC4-10 (data not shown). However, the murine leukocyte lines A20, P388D1, and T28 cells did not show any discernible change in the expression of ICAM-1 following cycloheximide treatment (Fig. 6), suggesting a cell type-specific effect of cycloheximide.

Measurement of De Novo ICAM-1 Protein Synthesis Under Cycloheximide Treatment

The upregulation of ICAM-1 expression on the cell surface by cycloheximide treatment described above can be explained by the 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 the increasing periods of time

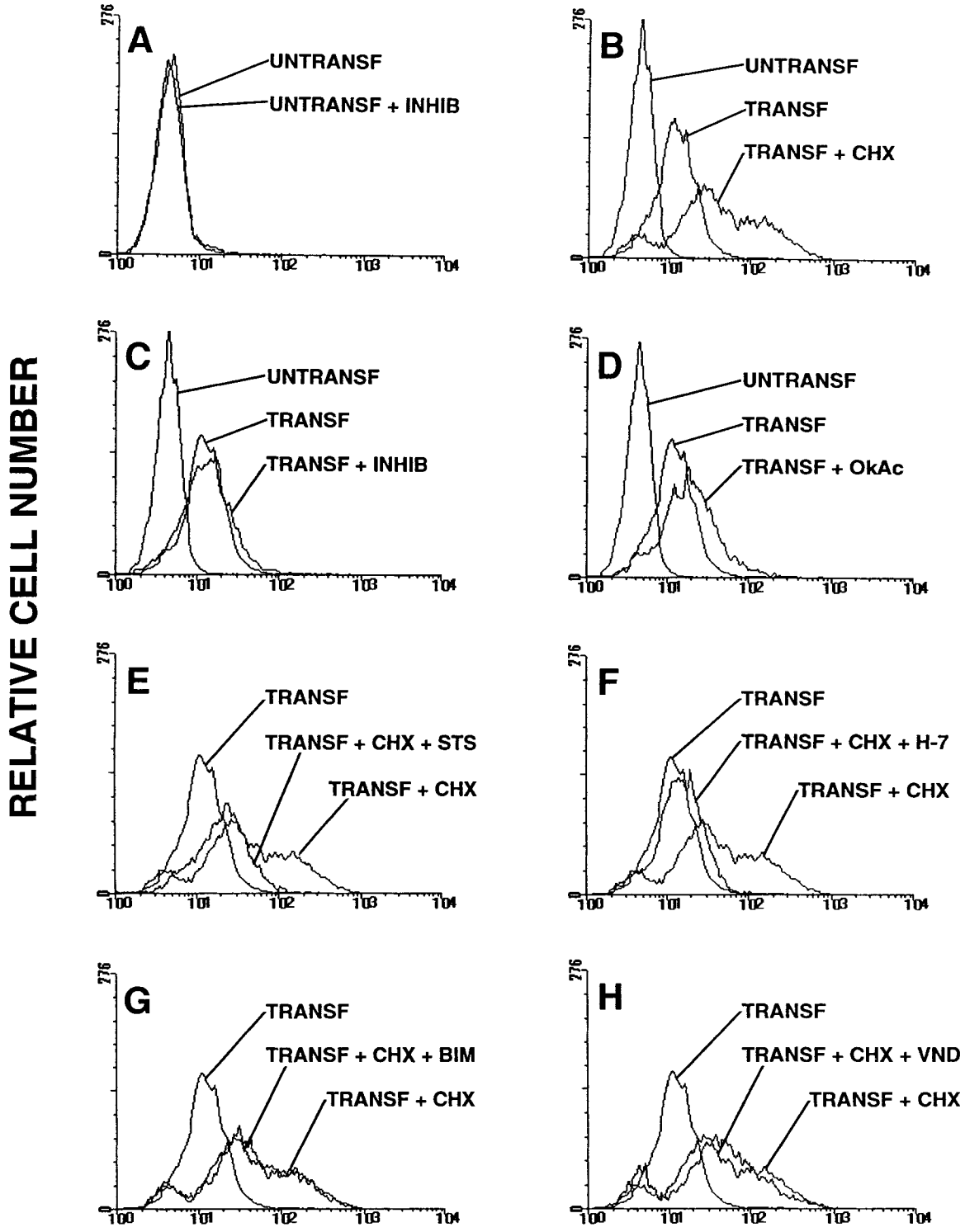

Fig. 5. Flow cytometric analysis of ICAM-1 cell surface expression. Cells were treated with kinase and phosphatase inhibitors in combination with cycloheximide for 12 h, then stained with YN1/1.7-FITC mAb and analyzed by flow cytometry. **A:** Effects of inhibitors including staurosporine, H-7, bisindolylmaleimide, 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 with 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.

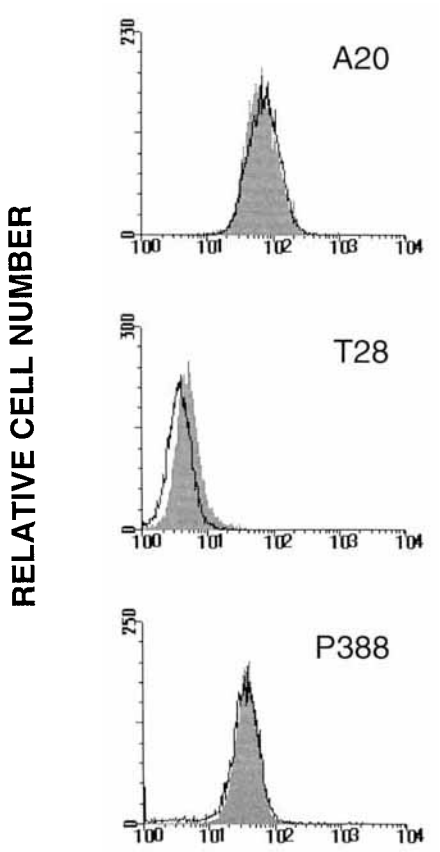

Fig. 6. Flow cytometric analysis of the effects of cycloheximide on ICAM-1 cell surface expression on A20, P388D1, and T28 cells. Cells were treated with cycloheximide for 12 h, then stained with YN1/1.7-FITC mAb and analyzed by flow cytometry. *Shaded histograms* represent the cell surface expression of ICAM-1 without the cycloheximide treatment; *lined histograms,* ICAM-1 cell surface expression with the cycloheximide treatment.

and then labeled with [^{35}S]-methionine for 2 h. The ICAM-1 proteins were 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. 7A). Concordantly, ICAM-1 protein synthesis was significantly reduced in the A20 cells (Fig. 7A). 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. 7B). However, cycloheximide did not diminish but rather increased ICAM-1 protein synthesis in SVEC4-10 and the L-ic-1 cells (Fig. 7A). 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 "escape" the translational inhibitory effect of cycloheximide. Because phosphorylation of certain proteins regulates general protein synthesis [Hershey, 1991], the effect of the ser/thr kinase and phosphatase inhibitors on total protein synthesis as well as their effect on cycloheximide treatment were examined. The inhibitors had no measurable effect on protein synthesis and did not influence the translational inhibition by cycloheximide (Fig. 7C), indicating that the effects of the ser/thr kinase and phosphatase inhibitors on ICAM-1 mRNA stabilization by cycloheximide is not due to their unexpected effects on the ability of cycloheximide to inhibit total protein synthesis.

DISCUSSION

Our present study has demonstrated that (1) cycloheximide stabilizes and superinduces ICAM-1 mRNA, but this effect does not require AUUUA repeats in the 3′UTR, (2) 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 effect of cycloheximide to stabilize ICAM-1 mRNA, (3) cycloheximide inhibits the overall protein synthesis but enhances the synthesis of the ICAM-1 protein and its expression on the cell surface in some, but not all, cell types. Cycloheximide is widely used as an eukaryotic protein synthesis inhibitor that acts by preventing the peptidyl transferase activity of the 60S ribosomal subunit. Although its ability to also stabilize and superinduce labile mRNAs is well known, the precise mechanisms are yet to be elucidated. In general, mRNA degradation is linked to translation [for review, see Jackson, 1993]. Two models have been proposed to explain this linkage. One states that the degradation of mRNA requires its translation. Therefore, the inhibition of translation results in the stabilization of mRNA. The second model states that a unique class of proteins that turn over rapidly are required for the degradation of certain highly labile mRNAs. According to this model, inhibition of general protein synthesis leads to down-regulation of these proteins that degrade rapidly, resulting in a quick down-regulation of the mRNA degradation. Our finding that cycloheximide stabilizes ICAM-1 mRNA without inhibiting the ICAM-1 protein synthe-

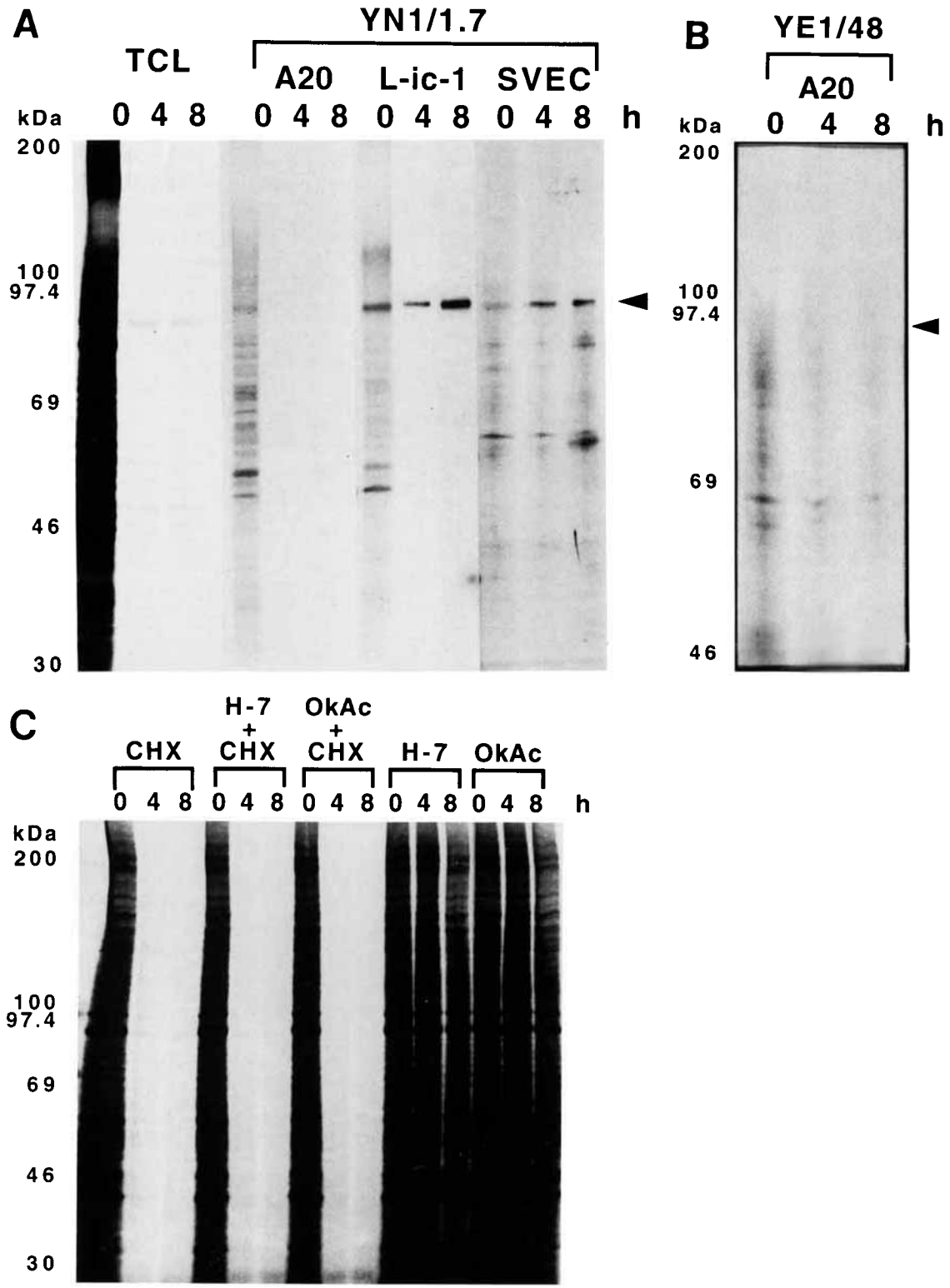

Fig. 7. Effect of cycloheximide on de novo ICAM-1 protein synthesis. **A:** Cells were treated with cycloheximide for the increasing periods of time. De novo ICAM-1 proteins that have incorporated [^{35}S]-methionine were immunoprecipitated from A20, L-ic-1, and SVEC4-10 cells with anti-ICAM-1 mAb and 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 arrowhead 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. The solid arrowhead marks 90 kDa. **C:** 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).

sis is incompatible with the first model and favors the second. It was quite unexpected that the synthesis of ICAM-1 determined by [^{35}S]-methionine labeling and immunoprecipitation was actually increased by cycloheximide treatment in two of the cell lines tested (transfected L cells and endothelial cells) while the overall protein synthesis in the same cells was profoundly inhibited. The increase 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 upon cycloheximide treatment. However, why ICAM-1 protein synthesis is not inhibited by cycloheximide in some cells while it effectively inhibits overall protein synthesis in the same cells is not known.

Our results also suggest that ser/thr phosphorylation of unknown proteins regulates 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 it and also augmented the cycloheximide effect. In contrast, other inhibitors including the protein tyrosine kinase inhibitors, genistein and tyrphostein, and 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 mechanism for the effect of cycloheximide on ICAM-1 mRNA stability. Based on the second model which proposes the presence of highly able 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. A simpler model of regulation of ICAM-1 mRNA degrading proteins by phosphorylation is not plausible, because staurosporine and H7 by themselves do not have any effect on ICAM-1 mRNA stability or induction, and they inhibit the effect of cycloheximide on ICAM-1 mRNA without affecting the inhibition of protein synthesis by cycloheximide. Furthermore, if mRNA degrading proteins were rapidly depleted by cycloheximide treatment, functional activation of such depleted proteins by dephosphorylation would not be able to destabilize ICAM-1 mRNA. Further studies to identify and characterize ICAM-1 mRNA degrading proteins will reveal the role of ser/thr phosphorylation in regulation of ICAM-1 mRNA stability. It is unknown whether the putative labile protein(s) itself degrades mRNA or 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 permits 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 it may provide new targets for modulating the course of inflammatory diseases.

ACKNOWLEDGMENTS

We thank Dr. Robert Cutler for his technical assistance in the metabolic labeling experiments and Dr. Vivienne Rebel for her kind help with the flow cytometric analysis. We also thank Dr. Graeme Dougherty for critically reading the manuscript. This work was supported by a grant from the Medical Research Council of Canada. M.O. is a recipient of the Medical Research Council of Canada studentship.

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