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Synthesis of p36 and p35 Is Increased When U-937 Cells Differentiate in Culture but Expression Is Not Inducible by Glucocorticoids

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p36 and p35 are distinct but related proteins that share many structural and biochemical features which were first identified as major substrates for protein-tyrosine kinases. Subsequently, both proteins have been shown to be Ca^{2+}-, phospholipid-, and F-actin-binding proteins that underlie the plasma membrane and are associated with the cortical cytoskeleton. Recent reports have claimed that these proteins function as lipocortins, i.e., phospholipase A_2 inhibitors that mediate the anti-inflammatory action of glucocorticoids. To investigate this possibility and to learn more about the functions of p36 and p35, we used human-specific anti-p36 and anti-p35 monoclonal antibodies to determine whether the expression or secretion of either protein was inducible by dexamethasone in the human U-937 myeloid cell line and in other human cell types. Additionally, we examined the levels of mRNA for both proteins. No effect of dexamethasone was observed on p36 or p35 expression at either the mRNA or protein level, nor were these proteins secreted under any of the culture conditions investigated. However, it was observed that in these cells the rate of synthesis and accumulation of both proteins was increased when the U-937 cells were induced to differentiate in culture to adherent macrophagelike cells. This offers a model system with which to study the control of p36 and p35 expression.

p36 and p35 were initially identified as major substrates for protein-tyrosine kinases (9, 14, 15, 20, 39, 52, 53, 58). Subsequently, their biochemical properties have been investigated in great detail, and this has led to their being termed calpactin I (p36) and calpactin II (p35) (21) or lipocortin I (p35) and lipocortin II (p36) (37). For clarity we will use the names p36 and p35 throughout. What has emerged from these studies is that the two proteins share many structural and functional features. First, both proteins bind Ca^{2+}, anionic phospholipids, and F-actin (12, 15, 19, 21, 22). Second, p35 and p36 are both abundant cellular proteins which are localized to the cortical cytoskeleton underlying the plasma membrane (24, 27, 47, 51). Third, p35 and p36 are both able to inhibit phospholipase A_2 (PLA_2) in vitro (11, 28, 36, 37, 49). Finally, the structure of p35 and p36 predicted from cDNA clones has revealed an overall structural similarity and ~50% amino acid identity (37, 42, 57, 62). Each protein has a short unique N-terminal sequence followed by four copies of a 75-amino-acid repeat. This repeat has been found in several other Ca^{2+}- and phospholipid-binding proteins with similar properties, and on this basis it has been proposed that this family of proteins be called annexins (18).

Two distinct functions for p36 and p35 have been proposed. First, their abundance, subcellular localization, and binding properties suggest that their function in vivo is in part structural and involves interaction with the plasma membrane and underlying protein components of the cortical skeleton. In this context, p36 has also been implicated in the secretion process by its ability to promote membrane vesicle fusion in vitro in the presence of Ca^{2+} (13).

Second, their ability to inhibit PLA_2 in vitro suggests that p36 and p35 might function to regulate PLA_2 in vivo. Evidence that PLA_2 activity can be regulated in vivo comes from the ability of glucocorticoid steroids, such as dexamethasone, to suppress inflammation (17). The inflammatory response is induced by leukotrienes, prostaglandins, and other eicosanoid compounds, which are synthesized from arachidonic acid released by PLA_2 hydrolysis of phospholipids. The mechanism by which glucocorticoids act to reduce arachidonic acid release is not fully understood, but there is evidence implicating a group of proteins that are secreted from macrophages in response to glucocorticoids and that can act on other cells to inhibit arachidonic acid release (2, 16, 17, 32, 35). These proteins have been termed lipocortins (previously known as either macrocortins or lipomodulins). Lipocortins are formally defined as proteins whose production and secretion is induced by glucocorticoids and which act extracellularly to inhibit eicosanoid production. Lipocortin activity can be measured as an inhibition of eicosanoid release both in cell culture and in model inflammation response systems in animals. There is some evidence that the activity of lipocortins can be modulated by phosphorylation (31, 33). In consequence, it is presumed that lipocortins act to regulate PLA_2 activity. However, there is no evidence from the biological assays that lipocortins act directly on PLA_2 itself rather than through a pathway leading to PLA_2.

On the assumption that lipocortins would act directly to inhibit PLA_2, Pepinksy and his colleagues purified a polypeptide with lipocortin activity from rat peritoneal exudate using a PLA_2 inhibition assay (49). This led to the isolation and subsequent cloning of lipocortin I, which was then shown to be identical to p35 (62). The level of lipocortin I mRNA was found to be increased in peritoneal cells following dexamethasone treatment, but no change in protein level

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was observed (62). Similar increases in lipocortin I mRNA were reported to occur when the U-937 human monocytic cell line was treated with dexamethasone (62). In keeping with the idea that this protein is a true lipocortin, recombinant lipocortin I has been shown to have anti-inflammatory activity in an in vivo assay (6). In a later study, a second polypeptide with lipocortin activity was purified that proved to be identical to p36 (37). Lipocortin II behaves similarly to lipocortin I in the PLA_2 inhibition assay (37).

Although these studies suggest a role for lipocortins I and II in the anti-inflammatory effects of steroids, there are a number of facts that are inconsistent with this notion. First, in many cell types p35 and p36 are very abundant proteins (21). Thus, steroids would not be expected to induce rapid changes in p35 or p36 levels, although secretion of preformed protein could occur in response to steroids. Second, p35 and p36 have been shown to inhibit PLA_2 by an indirect mechanism involving sequestration of phospholipid substrate rather than by direct interaction with the enzyme (11, 28). Third, neither p35 nor p36 has a conventional signal peptide for entry into the cellular secretion pathway (37, 42, 57, 62), which precludes secretion of these proteins by a conventional mechanism.

In an attempt to understand more about the function of p36 and p35 and to determine whether their expression could be modulated by steroids in a fashion consistent with a role as lipocortins, we have examined their expression in U-937 cells under a variety of conditions. The initial reason for choosing this cell line was that Wallner et al. (62) isolated a lipocortin I (p35) cDNA from a U-937 cell library and stated that there was an increase in the level of p35 mRNA when the cells were treated with dexamethasone. Further, Hattori et al. (30) reported that treatment of U-937 cells with dexamethasone induced lipocortin production.

A second reason for examining U-937 cells was that they can be induced to differentiate in vitro from an immature monocytic phenotype into adherent macrophagelike cells by the addition of phorbol esters (48, 61). Preliminary experiments demonstrated that the levels of p36 and p35 in nondifferentiated U-937 cells were much lower than in cultured fibroblasts or epithelial cells. We were interested in determining whether alteration of the culture conditions or induction to differentiate could alter the expression of p36 or p35 and thereby provide a model system with which to investigate their possible function.

MATERIALS AND METHODS

Cells. U-937 (61) and HL-60 (8) cells were obtained from Richard Mitchell (The Salk Institute) and cultured in RPMI 1640 containing 10% (vol/vol) fetal bovine serum (FBS). To induce differentiation, cells were plated at 2×10^5/ml in RPMI 1640 plus 10% FBS, left for 16 h, and then treated with either 50 ng of 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (Sigma Chemical Co., St. Louis, Mo.) per ml dissolved in dimethyl sulfoxide (DMSO) at 50 μg/ml, 1 μM retinoic acid (trans-retinoic acid; Calbiochem-Behring, La Jolla, Calif.) dissolved in ethanol at 1 mM, or 1.25% (vol/vol) DMSO (Sigma) for the required period of time. Differentiation status was monitored by morphological changes and division rates. Differentiated or undifferentiated cells were treated with dexamethasone (Sigma) dissolved in H_2O as indicated in the figure legends. AG1523 cells obtained from NIA Aging Cell Repository, Institute for Medical Research, Camden, N.J., were cultured in Dulbecco-Vogt modified Eagle medium containing 10% FBS.

Monoclonal antibodies. The anti-human p36 monoclonal antibodies (MAbs) (D1/274 and B1/107) have been described in Isacke et al. (38). The anti-human p35 MAb (74/3) was generated in a manner identical to that described for D1/274 and B1/107 (38). Essentially, BALB/c mice were immunized with AG1523 human fibroblasts, the spleens from immune mice were removed and fused with Sp2/0 myeloma cells, and the supernatants from the resulting hybridomas were screened for their ability to immunoprecipitate protein from ^{125}I-labeled or [^{35}S]methionine-labeled AG1523 cells. The 74/3 hybridoma was cloned by limiting dilution, and the resulting stable cell line was shown to secrete immunoglobulin of the IgG1 subclass.

Labeling and immunoprecipitation of p36 and p35. For long-term labeling with [^{35}S]methionine, cells were washed two times with methionine-free RPMI 1640 and then suspended at 1×10^6 cells per ml of methionine-free RPMI 1640 supplemented with 5% (vol/vol) RPMI 1640, 5% (vol/vol) dialyzed FBS, and 300 μCi of [^{35}S]methionine (>1,000 Ci/mmol) (Amersham Corp., Arlington Heights, Ill.). After 6 or 16 h, cells were pelleted, the supernatants were retained, and the cells were lysed in RIPA buffer (60). The supernatants were either immunoprecipitated directly or added to an equal volume of 2× RIPA buffer before immunoprecipitation. For pulse-labeling, cells were cultured for the indicated periods of time, gently pelleted, and suspended in methionine-free RPMI 1640 containing [^{35}S]methionine. After 30 min, the cells were pelleted and lysed in RIPA buffer. Cell lysates were clarified by centrifugation, and a portion of each was precipitated with trichloroacetic acid to determine the amount of [^{35}S]methionine incorporated into protein. Equal amounts of precipitable [^{35}S]methionine counts per minute were used for each immunoprecipitation. Lysates were precleared for 30 min with 1% (vol/vol) fixed *Staphylococcus aureus* bacteria (Pansorbin; Calbiochem-Behring). Immunoprecipitations were then performed as described by Sefton et al. (60) and resolved on 15% sodium dodecyl sulfate-polyacrylamide gels. Where indicated, gels were enhanced with diphenyloxazole before being dried and exposed to presensitized Kodak XAR X-ray film. Peptide mapping was done as described previously (1).

Western blotting. Immunoblotting of p36 was performed exactly as described by Gould et al. (25) using a polyclonal anti-chicken p36 serum (10).

Northern (RNA) blot analysis. Total cellular RNA was isolated by the guanidine thiocyanate method (5). Nitrocellulose (Schleicher & Schuell, Inc., Keene, N.H.) blots were made from formaldehyde gels (1 or 1.4% agarose) and hybridized as described by Maniatis et al. (44). The blots were washed to a final stringency of 0.2× SSPE at 42°C. Hybridization probes were labeled by nick translation (44). The p36 probe was a 733-base-pair *Hin*dIII-*Xba*I fragment from the murine cDNA clone isolated by Saris et al. (57). A human p35 clone was isolated by screening a HeLa cell cDNA library provided by Steven Hanks (The Salk Institute) (29) with a 32-mer complementary to nucleotides −5 to 27 of p35 as reported by Wallner et al. (62). The p35 probe used for Northern analysis was the 1,301-base-pair *Aat*II-*Eco*RI fragment, which encompasses nearly the entire human p35 coding sequence. A 168-base-pair *Bam*HI-*Pvu*II fragment from the coding region of human metallothionein II_A was provided by Michael Karin (University of California, San Diego) (41). For densitometry of Northern blots, β-actin mRNA levels were used as an internal standard. The probe was the entire cDNA insert described by Cleveland et al. (7). Densitometric scanning of autoradiograms and signal inte-

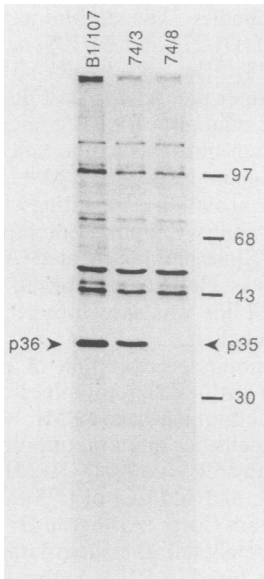

FIG. 1. Immunoprecipitation of human p35 and p36. A 35-mm dish of A431 cells was labeled for 16 h in 1 ml of methionine-free Dulbecco-Vogt modified Eagle medium containing 5% dialyzed FBS, 5% Dulbecco-Vogt modified Eagle medium, and 300 μCi of [^{35}S]methionine. Cell lysates (in 1 ml of RIPA buffer) were prepared, divided into three parts, and immunoprecipitated as described in Materials and Methods. The gel was exposed for 7 h. Markers are in kilodaltons. B1/107, Anti-human p36; 74/3, anti-human p35; and 74/8, an irrelevant MAb derived from the same fusion as 74/3.

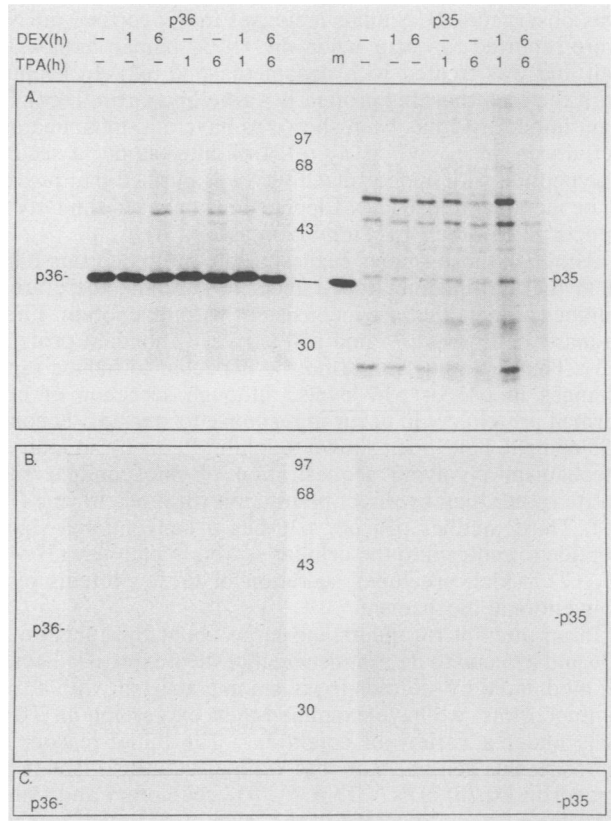

FIG. 2. Short-term treatment of U-937 cells with TPA or dexamethasone or both. U-937 cells (1.3×10^6) were incubated overnight in 1.5 ml of RPMI 1640 plus 10% FBS. The next day the cells were labeled in 1 ml with 300 μCi of [^{35}S]methionine for 6 h as described in Materials and Methods. Where indicated, 10 μM dexamethasone and 50 ng of TPA per ml were added to the cells singly or in combination for either the whole labeling period or for the last hour. Culture supernatants and cell lysates (in 1 ml of RIPA buffer) were prepared, divided into two, and immunoprecipitated either with MAb D1/274 (anti-p36) or MAb 74/3 (anti-p35) as outlined in Materials and Methods. Enhanced gels were exposed as follows: A, Cell lysates (14 h); B, culture supernatants (14 h); C, same as panel B except exposure time was 6 days. The lane (m) to the left of the p35 immunoprecipitations is a control p36 immunoprecipitation included to show the relative mobilities of the two proteins in this gel system. Markers are in kilodaltons.

gration was done with a Quick Scan RD (Helena Laboratories) within a predetermined linear range of response.

RESULTS

Characterization of MAb 74/3 (anti-human p35). A MAb against human p35 (MAb 74/3) was isolated by screening hybridoma supernatants for their ability to immunoprecipitate a 35-kilodalton protein from human cell lines. As shown by analysis of immunoprecipitates made from A431 cells, the protein recognized by MAb 74/3 migrates on reducing sodium dodecyl sulfate-polyacrylamide gels with a slightly slower mobility than human p36 (Fig. 1). This mobility is characteristic of p35 as shown by Glenney et al. (21, 24) and is predicted from a sequence comparison of the two proteins (37, 57). To characterize this protein further, the bands immunoprecipitated by the anti-p36 MAb (MAb B1/107) and MAb 74/3 were excised from the gel shown in Fig. 1, eluted, and digested with trypsin. The resulting [^{35}S]methionine-labeled peptides were resolved in two dimensions and compared with [^{35}S]methionine-labeled tryptic peptides from human p35 immunoprecipitated by a cross-reactive rabbit polyclonal serum generated against bovine p35 (24). The pattern of ^{35}S-labeled peptides from the protein immunoprecipitated by MAb 74/3 was identical to that of the p35 immunoprecipitated with anti-p35 polyclonal serum (data not shown). Furthermore, when the pattern of p35 tryptic peptides was compared with that of p36, all were completely different except for one common methionine-containing tryptic peptide (data not shown). A single common methionine-containing peptide, Ile-Met-Val-Ser-Arg, is predicted from a comparison of the protein sequences of p35 (residues 299 to 303) and p36 (residues 291 to 295) (37, 57).

MAb 74/3 does not cross-react with chicken, mouse, rat, or bovine p35, nor does it detect denatured human p35 as assayed by immunoblotting. Since MAb 74/3 does not bind directly to *S. aureus* bacteria, in all the immunoprecipitation experiments described here a rabbit anti-mouse immunoglobulin second-layer antibody was used. When used at saturating levels, MAb 74/3 immunoprecipitated the same amount of p35 from A431 cells as a polyclonal rabbit antiserum raised against bovine lung p35 (24). For this reason, we believe that MAb 74/3 recognizes the majority of the cellular p35 and not a select subpopulation.

Dexamethasone does not induce the synthesis or secretion of p36 or p35. A large number of experiments were performed to determine whether the treatment of U-937 cells with the synthetic glucocorticoid dexamethasone had any effect on the levels, rates of synthesis, or secretion of p36 and p35. In the experiment shown in Fig. 2, U-937 cells were labeled for a total of 6 h with [^{35}S]methionine, and dexamethasone or TPA or both were present for the whole labeling period or for the last hour. Under such conditions, neither protein was detected in the culture supernatants (panel B) even if the gels

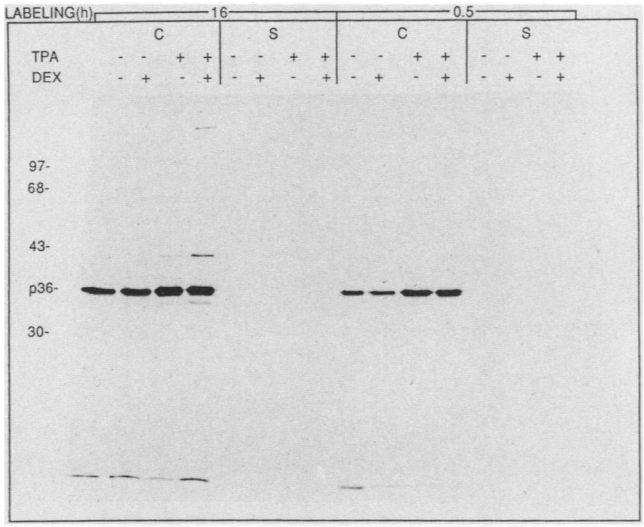

FIG. 3. Long-term dexamethasone treatment of U-937 cells. U-937 cells (1×10^6) were cultured in 1 ml of methionine-free RPMI 1640 containing 5% RPMI 1640–5% dialyzed FBS together with 10 μM dexamethasone and 20 ng of TPA per ml singly or in combination where indicated. During this incubation, half of the samples contained 300 μCi of [^{35}S]methionine. After 16 h, the unlabeled cells were pelleted and suspended in 150 μl of methionine-free RPMI 1640 containing 150 μCi of [^{35}S]methionine for 30 min. Culture supernatants and cell lysates (in 0.5 ml of RIPA buffer) were prepared and immunoprecipitated with MAb D1/274 (anti-p36) as described in Materials and Methods. Enhanced gels were exposed for 18 h. C, Cells; S, supernatants. Markers are in kilodaltons.

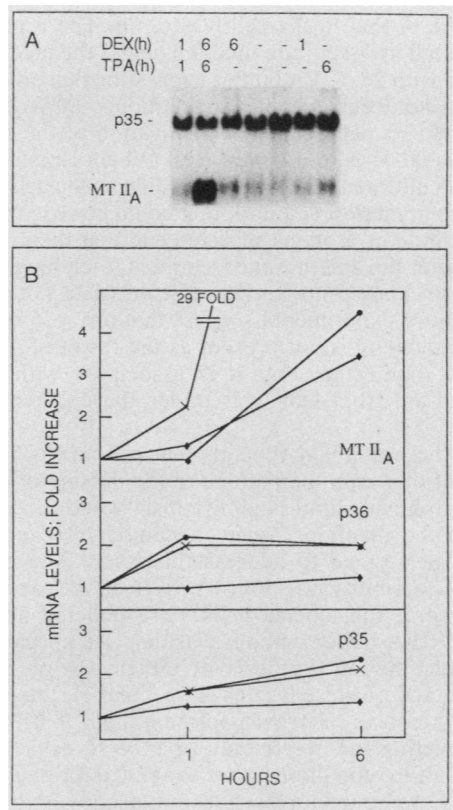

FIG. 4. Northern blot analysis of U-937 cells treated with dexamethasone or TPA or both. U-937 cells were incubated overnight in RPMI 1640 containing 10% FBS. The following day, 10 μM dexamethasone or 50 ng of TPA per ml or both were added where indicated for 1 or 6 h. The cells were then lysed in guanidine thiocyanate for preparation of whole-cell RNA. RNA (10 μg) was electrophoresed on formaldehyde gels and transferred for Northern analysis as described in Materials and Methods. Two identical blots were incubated with probes either for p36 or for p35 and metallothionein II$_A$. (A) Blot of U-937 RNA probed simultaneously for p35 and metallothionein II$_A$ expression. Exposure time was 4 days at −70°C with an intensifying screen. (B) Densitometric scan of p36, p35, and metallothionein II$_A$ RNA levels in U-937 cells treated with TPA or dexamethasone or both. Sixteen-hour exposures of the blots were used for densitometry. The fold increase was normalized against the same blots probed subsequently with actin cDNA. Symbols: ◆, dexamethasone; ●, TPA; ×, both.

were exposed for a 10-fold-longer period of time (panel C). In addition, there was no alteration in the amounts of labeled p36 or p35 immunoprecipitated from these cells. To determine whether our inability to detect such changes was due to our culture or labeling conditions, many experimental variables were examined. For example, U-937 cells were either labeled to equilibrium for 16 h with [^{35}S]methionine in the presence or absence of dexamethasone, or they were incubated for 16 h with dexamethasone and then pulse-labeled with [^{35}S]methionine for 30 min prior to immunoprecipitation with the p36-specific MAb D1/274 (Fig. 3). The former condition provides a measure of the total amount of immunoprecipitable p36 present, whereas the latter reflects the rate at which it is being synthesized. Again, there was no detectable change in the expression of p36, and no p36 was detected in the culture supernatants (Fig. 3). We have performed these experiments under different labeling conditions, with variable amounts of dexamethasone, with or without TPA, and for different lengths of dexamethasone treatment time. No effect of dexamethasone treatment on either p36 or p35 expression was ever observed.

To confirm that glucocorticoids have no effect on the expression of the p36 and p35 genes in U-937 cells, we examined the mRNA levels in cells after dexamethasone treatment. Incubation of the cells for 1 or 6 h with dexamethasone or TPA or both had little effect on the levels of p35 mRNA (Fig. 4A). Densitometric scans of Northern blots showed that neither p36 nor p35 mRNA levels change in response to dexamethasone (Fig. 4B). TPA caused a slight increase in the levels of both mRNAs, but dexamethasone plus TPA showed no increase over TPA alone. As a control, these blots were also probed for metallothionein II$_A$ mRNA expression. Glucocorticoids are known to induce metallothionein in a number of different cell types (40, 54), and in U-937 cells we found that the level of metallothionein II$_A$ mRNA increased ~3-fold when the cells were treated for 6 h with dexamethasone and ~29-fold when the cells were treated for 6 h with both dexamethasone and TPA. Therefore, the failure of dexamethasone to induce p36 and p35 expression is not due to these cells being refractory to the effects of glucocorticoids.

It should be noted that treatment of U-937 cells for long periods of time (>16 h) with high concentrations of dexamethasone (10 μM) did occasionally result in the appearance of p36 and p35 in the culture supernatants. Extensive experiments with p36 subsequently demonstrated that this material is almost certainly associated with cell debris and results from cell death due to the prolonged steroid treatment. The evidence that cell death rather than some active secretion or exocytosis mechanism is responsible for p36 in the culture medium is as follows. (i) Under these conditions, p36 was found in the culture medium in only 20% of the experiments. (ii) The level of p36 in the medium was variable and always

less than 5% of that in the cell lysate. (iii) The protein was never detected in the culture medium unless the medium was first treated with 2× RIPA buffer, suggesting that any protein present was not freely soluble. In addition, >90% of the p36 present could be pelleted by centrifugation of the supernatants at 10,000 × *g* for 10 min. (iv) When samples of the ^{35}S-labeled culture medium were run directly on gels and not subjected to immunoprecipitation, it could be seen that there was a multitude of proteins secreted and that their levels all increased after dexamethasone treatment, including proteins such as actin known not to be secreted (data not shown). Together these experiments suggest that any p36 or p35 in the culture medium is not present as the result of a specific mechanism, but rather that it is associated with cellular debris resulting from cell lysis under these harsh culture conditions.

Finally, the possibility remains that p36 and p35 may be secreted but that they then attach to the outside of the cells due to their phospholipid-binding capacity and the presence of Ca^{2+} in the medium. Again, a number of experiments have been performed to address this issue. The evidence against this possibility is as follows. (i) In all the experiments described here, the labeled cells were pelleted and lysed directly in RIPA buffer without washing. As no increase in the level or rate of synthesis of either p36 or p35 was observed in any of these lysates (Fig. 2 and 3), this suggests that there is not an external pool of protein that increases with dexamethasone treatment. (ii) U-937 cells under a variety of culture conditions were examined for external p36 and p35 by immunofluorescence microscopy. No protein was ever detected unless the cells were first attached to cover slips and permeabilized to reveal intracellular protein (data not shown).

As well as examining the effects of dexamethasone in U-937 cells, we also looked at a number of other cell lines, including AG1523 human fibroblasts, HEp-2 human epithelial cells, A431 carinoma cells, and HL-60 cells. Again, no alteration in the levels or synthesis of p36 or p35 with dexamethasone treatment was detected in these cells, nor were these proteins detected in the culture supernatant. For example, no change in the level of p36 was observed when AG1523 fibroblasts were labeled for 16 h with [^{35}S]methionine in the presence of either 1 or 10 μM dexamethasone or 50 ng of TPA per ml (Fig. 5). In addition, we examined whether p36 expression was dexamethasone responsive in differentiated U-937 cells. Treatment of U-937 cells causes them to differentiate into macrophagelike cells. As the cells differentiate, there is an increase in the rate of synthesis and the level of p36 (see below), but dexamethasone treatment of the parental cells or their differentiated progeny for 16 h did not alter p36 expression (Fig. 3).

The effects of dexamethasone treatment on the phosphorylation of both proteins was also examined, and no detectable change was ever observed (data not shown).

Modulation of p36 and p35 expression in differentiating U-937 cells. U-937 cells are an immature monocytic cell line that can be induced to differentiate into macrophagelike cells by treatment with TPA (45, 48). To determine whether the levels of p36 or p35 could be modulated during differentiation, U-937 cells were treated with 20 ng of TPA per ml for up to 48 h, pulse-labeled for 30 min with [^{35}S]methionine, and then immunoprecipitated with either anti-p36 or anti-p35 MAb. The rates of both p36 and p35 synthesis increased an estimated fivefold between 8 and 16 h (Fig. 6). This corresponds to the time when the cells become adherent to the substratum and show morphological signs of differentiation.

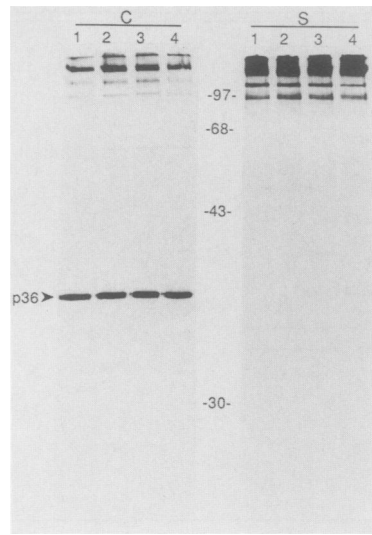

FIG. 5. Dexamethasone and TPA treatment of AG1523 cells. Dishes (35 mm) of near-confluent AG1523 cells plated the previous day were labeled for 16 h in 1 ml of methionine-free RPMI 1640 containing 5% RPMI 1640, 5% dialyzed FBS, 450 μCi of [^{35}S] methionine, and the following additions. Lanes: 1, no addition; 2, 10 μM dexamethasone; 3, 1 μM dexamethasone; 4, 50 ng of TPA per ml. Culture supernatants and cell lysates (in 1 ml of RIPA buffer) were prepared and immunoprecipitated with MAb D1/274 (anti-p36) as described in Materials and Methods. Exposure time was 4 days. C, Cells; S, supernatants. Markers are in kilodaltons.

This increase in the rate of p36 and p35 synthesis was paralleled by an increase in the level of protein as detected by immunoprecipitation from cells that have been labeled to equilibrium (Fig. 3) or by Western blotting of protein (data not shown) and by an increase in the level of mRNA (Fig. 4).

Modulation of p36 and p35 in differentiating HL-60 cells. Finally, we wished to determine whether the modulation of p36 and p35 during U-937 cell differentiation was found in

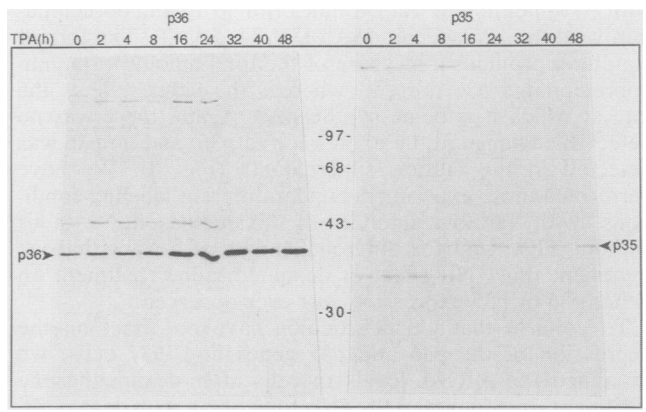

FIG. 6. Long-term treatment of U-937 cells with TPA. U-937 cells (2×10^5 per well) were incubated in 24-well dishes in 1.5 ml of RPMI 1640 containing 5% FBS. Twenty ng of TPA per ml was added at intervals to the wells for the indicated lengths of time. After 48 h, the cells were pelleted, suspended in 150 μl of methionine-free RPMI 1640 containing 250 μCi of [^{35}S]methionine, and cultured for 30 min. Lysates (in 0.5 ml of RIPA buffer) were immunoprecipitated with either MAb D1/274 (anti-p36) or MAb 74/3 (anti-p35) as described in Materials and Methods. The enhanced gels were exposed for 12 h (p36) or 7 days (p35). Markers are in kilodaltons.

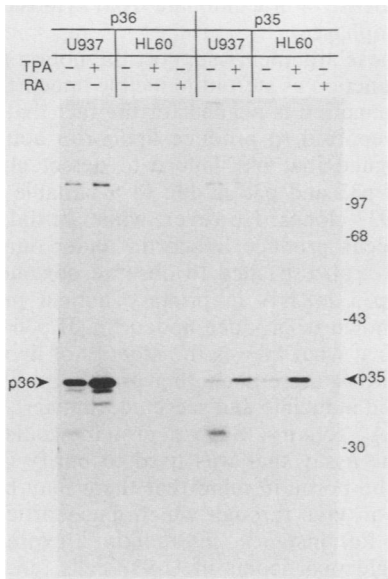

FIG. 7. Long-term treatment of U-937 and HL-60 cells with TPA and retinoic acid. U-937 or HL-60 cells were treated exactly as described in the legend to Fig. 6 except that 1 μM retinoic acid or 20 ng of TPA per ml was added for 16 h before the cells were pulse-labeled with [^{35}S]methionine for 30 min. Exposure time was 3 days.

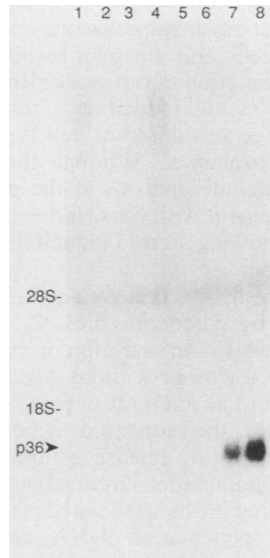

FIG. 8. Northern blot analysis of U-937 and HL-60 cells induced to differentiate in culture. Cells were incubated overnight in RPMI 1640 plus 10% FBS. Retinoic acid (1 μM) or DMSO (1.25%) was added for the indicated periods of time, and cells were then lysed in guanidine thiocyanate and RNA prepared for Northern analysis as described in Materials and Methods. The blot was probed with p36 cDNA. Exposure time was 5 days at −70°C with an intensifying screen. Lanes 1 through 6, RNA from HL-60 cells; lanes 7 and 8, RNA from U-937 cells. Treatments were as follows. Lanes: 1, no treatment (1 day); 2, no treatment (5 days); 3, DMSO (1 day); 4, DMSO (3 days); 5, DMSO (5 days); 6, retinoic acid (3 days); 7, no treatment (3 days); 8, DMSO (3 days).

other cell systems and whether it might be a specific effect of TPA treatment. In our earlier experiments we had examined the effects of long-term TPA treatment of several nonhematopoietic cell lines and found that TPA treatment of AG1523, HEp-2, and A431 cells for 16 to 24 h had no effect on the levels of p36 or p35 (Fig. 5), although these cells respond to TPA as determined by other biological assays. In contrast to these adherent cell lines, HL-60 cells, like U-937 cells, can also be induced to differentiate from a promyelocytic phenotype to macrophagelike cells with TPA treatment. However, unlike U-937 cells, HL-60 cells are bipotential and can also be induced to differentiate into granulocytes by treatment with retinoic acid or DMSO (8, 45, 55). It can be seen again that treatment of U-937 cells with TPA resulted in an increase in the rate of p36 and p35 synthesis (Fig. 7). In a similar manner, it was found that the rate of p35 synthesis increased in HL-60 cells induced to differentiate into macrophages by exposure to TPA. In contrast, retinoic acid treatment, which results in differentiation into granulocytes, slightly decreased the rate of p35 synthesis. Curiously, we were unable to detect p36 in either differentiated or undifferentiated HL-60 cells.

To determine whether this regulation of p35 and p36 expression was at the level of mRNA accumulation, Northern analysis of these cells was performed. No p36 mRNA was detectable in HL-60 cells treated with DMSO or retinoic acid (Fig. 8). In contrast, p36 mRNA was detected in U-937 cells, and the amount was found to increase in cells treated with DMSO, which results in the differentiation of U-937 cells into macrophages (46). This also shows that induction of p36 is not specific to TPA.

In general, increases in the rate of p36 and p35 synthesis as detected by immunoprecipitation were always paralleled by an increase in the level of mRNA. Furthermore, when it was examined, it was found that the level of p36-associated light-chain (p11, also known as p10 or calpactin I light-chain) (19, 23, 56) mRNA increased in parallel with p36 (calpactin I heavy-chain) mRNA, suggesting that they are coordinately regulated in these cells.

We have subsequently examined the expression of p36 in HL-60 cells obtained from other sources. In these lines, a low level of p36 was sometimes detected, and this increased when the cells were induced to differentiate into macrophages. However, the level of p36 was always substantially lower than the level of p35 (data not shown).

DISCUSSION

Dexamethasone has no effect on the synthesis or secretion of p36 or p35 in U-937 cells. To determine whether dexamethasone can induce the synthesis or secretion of either p36 or p35 in U-937 cells, we examined the levels of protein, the rate of protein synthesis, and the level of mRNA in these cells. We were not able under a variety of conditions to observe any effect of dexamethasone treatment on the expression of either of these proteins, nor did dexamethasone induce their secretion. Our results and other published data indicate that these proteins may not function as lipocortins in vivo. The evidence is as follows.

(i) By definition, lipocortins are glucocorticoid inducible (2, 16, 17). We have been unable to find any evidence for the dexamethasone inducibility of p36 or p35 expression either in U-937 cells or in a variety of other cell lines examined (Fig. 1 to 5). Wallner et al. (62) showed that the level of p35 mRNA is increased in rat peritoneal cells treated with dexamethasone and stated, but did not show, that the same was true for U-937 cells treated with dexamethasone. However, they were unable to find any increase in the level of p35 protein in either cell type as detected by Western blotting

(62). There is no obvious explanation for the discrepancy between this effect of dexamethasone on the level of p35 mRNA in U-937 cells and our own results. However, subsequent to the completion of our work, Bronnegard et al. (3) reported that p35 is not inducible at the mRNA level by dexamethasone in seven different cell types, including primary human macrophages. Although they examined only p35 and did not include analysis at the protein level, their results are in agreement with ours. Indeed, as yet there have been no reports showing steroid inducibility of either p35 or p36 in any cell type.

(ii) By definition, lipocortins are secreted, and this secretion is enhanced by glucocorticoids (2, 34, 35). We have never detected any specific secretion or exocytosis of p36 or p35 in cells cultured with or without dexamethasone. In the few cases in which small amounts of protein were detected in the culture medium, this appeared to be the result of cell death and the nonspecific release of intracellular proteins. p36 and p35 found under such circumstances were not freely soluble but appeared to be associated with cellular debris. Furthermore, the sequence of cDNA clones encoding p36 and p35 does not reveal the N-terminal hydrophobic region or signal peptide usually required for transmembrane trafficking (37, 57, 62). Thus, neither p36 nor p35 can gain access to the cellular secretion pathway by conventional means. Moreover, there have been no other reports showing the secretion of either protein.

(iii) The proposal that p36 and p35 function as lipocortins in vivo was initially based on the observation that both proteins can inhibit PLA_2 in vitro. However, Davidson et al. (11) and Haigler et al. (28) have provided evidence that this activity in vitro is due to the ability of p36 and p35 to bind phospholipid in the presence of Ca^{2+}, which results in substrate competition with PLA_2 (i.e., they bind to the PLA_2 substrates) rather than true enzyme inhibition (binding to the enzyme directly and thereby blocking its activity). For example, Davidson et al. (11) performed titration experiments and showed that p36 and p35 did not inhibit the activity of PLA_2 when the phospholipids were present in excess. Because p36 and p35 apparently act by a mechanism of substrate competition, this raises doubts as to whether they could block the activity of PLA_2 in vivo by a similar mechanism.

(iv) The activity of lipocortins is supposedly regulated by phosphorylation so that when phosphorylated they no longer inhibit PLA_2 (31, 33). Although it has been well established that both p36 and p35 can be phosphorylated on serine and tyrosine residues (9, 14, 15, 26, 39, 52, 58), only a small percentage (up to 5%) of molecules become phosphorylated at any one time. There is some evidence that phosphorylation of p35 and p36 alters their phospholipid-binding properties, although the effects on the two proteins seem to be contrary (50, 59). In preliminary experiments we were not able to detect any changes in phosphorylation of these proteins in dexamethasone-treated cells (data not shown).

(v) Experiments have been performed to determine whether purified p36 or p35 can function in vivo in anti-inflammatory assays. The results from this type of analysis are contradictory. Cirino et al. (6) have reported that bacterially expressed p35 (lipocortin I) will inhibit eicosanoid release when used in a guinea pig lung infusion system. However, the authors state that they are not able in this assay to distinguish between p35 acting as a substrate competitor or as an enzyme inhibitor. Northup et al. (48a) have found that purified placental p35 does not act as an anti-inflammatory agent in a paw edema assay, nor will it inhibit the release of arachidonate from zymosan-stimulated mouse macrophages.

In sum, these arguments support the notion that p35 and p36 do not function as steroid-inducible lipocortins. If this is true, an explanation is needed for the fact that U-937 cells have been reported to produce lipocortin activity (30). It could be argued that our failure to detect glucocorticoid induction of p35 and p36 is due to a variable response of different U-937 clones. However, while we did not confirm that U-937 cells produce lipocortin under our conditions, Bronnegard et al. (3) failed to observe dexamethasone induction of p35 mRNA in primary human macrophages, which are known to produce lipocortin. If p36 and p35 are not lipocortins, what then is the identity of lipocortin? One possibility is that there is another protein(s), which is both glucocorticoid inducible and secreted, that acts indirectly to regulate PLA_2 activity. Such a protein would have been missed in the assay that was used to purify lipocortin. It should also be borne in mind that there may be additional mechanisms in vivo through which glucocorticoids can inhibit PLA_2. For instance, interleukin 1 expression is decreased by glucocorticoids in U-937 cells, and this would have an anti-inflammatory effect (43).

Function of p36 and p35 other than as lipocortins. The increase in p36 and p35 expression in U-937 cells induced to differentiate into macrophages supports the suggestion that they might play a role in membrane movement or cell attachment or both by interacting with the cytoskeleton and overlying plasma membrane. Unlike their precursors, which grow in suspension culture, macrophages are adherent cells which exhibit extensive membrane fluidity in the form of ruffling and exocytosis and which respond to chemoattractants. Support of this function for these proteins has come from studies by Carter et al. (4). They have demonstrated that limb buds from 5-day-old chicks express very low levels of p36 protein but that if these are cultured in vitro for 48 h, there is a dramatic increase in the level of protein. Furthermore, this increase is dependent upon the cells being grown on a substratum, as culture in semisolid medium does not result in increased p36 expression.

Here we have described the isolation of a MAb specific for human p35 (MAb 74/3). The availability of reagents (see also references 38 and 63) and molecular probes should allow a detailed investigation into the control of p36 and p35 expression and help to elucidate their function in vivo. The finding of an in vitro culture system in which the expression of these proteins is regulated will be useful for future experiments.

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LITERATURE CITED

1. **Beemon, K., and T. Hunter.** 1978. Characterization of Rous sarcoma virus gene product synthesized in vitro. J. Virol. **28**:551–556.
2. **Blackwell, G. J., R. Carnuccio, M. Di Rosa, R. J. Flower, L.**

Parente, and P. Persico. 1980. Macrocortin: a polypeptide causing the anti-phospholipase effect of glucocorticoids. Nature (London) **287**:147–149.
3. **Bronnegard, M., O. Andersson, D. Edwall, J. Lund, G. Norstedt, and J. Carlstedt-Duke.** 1988. Human calpactin II (lipocortin I) messenger ribonucleic acid is not induced by glucocorticoids. Mol. Endocrinol. **2**:732–739.
4. **Carter, V. C., A. R. Howlett, G. S. Martin, and M. J. Bissell.** 1986. The tyrosine phosphorylation substrate p36 is developmentally regulated in embryonic avian limb and is induced in cell culture. J. Cell Biol. **103**:2017–2024.
5. **Chirgwin, J. M., A. E. Pryzbyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.
6. **Cirino, G., R. J. Flower, J. L. Browning, L. K. Sinclair, and R. B. Pepinsky.** 1987. Recombinant human lipocortin 1 inhibits thromboxane release from guinea-pig isolated perfused lung. Nature (London) **328**:270–272.
7. **Cleveland, D. W., M. A. Lopata, R. J. MacDonald, N. J. Cowan, W. J. Rutter, and M. W. Kirschner.** 1980. Number and evolutionary conservation of α- and β-tubulin and cytoplasmic β- and α-actin genes using specific cloned cDNA probes. Cell **20**:95–105.
8. **Collins, S. J., R. C. Gallo, and R. E. Gallagher.** 1977. Continuous growth and differentiation of human myeloid leukemic cells in suspension culture. Nature (London) **270**:347–349.
9. **Cooper, J. A., and T. Hunter.** 1981. Similarities and differences between the effects of epidermal growth factor and Rous sarcoma virus. J. Cell Biol. **91**:878–883.
10. **Cooper, J. A., and T. Hunter.** 1983. Identification and characterization of cellular targets for tyrosine protein kinases. J. Biol. Chem. **258**:1108–1115.
11. **Davidson, F. F., E. A. Dennis, M. Powell, and J. R. Glenney.** 1987. Inhibition of phospholipase A_2 by "lipocortins" and calpactins. J. Biol. Chem. **262**:1698–1705.
12. **De, B. K., K. S. Misono, T. J. Lukas, B. Mroczkowski, and S. Cohen.** 1986. A calcium-dependent 35-kilodalton substrate for epidermal growth factor receptor/kinase isolated from normal tissue. J. Biol. Chem. **261**:13784–13792.
13. **Drust, D. S., and C. E. Creutz.** 1988. Aggregation of chromaffin granules by calpactin at micromolar levels of calcium. Nature (London) **331**:88–91.
14. **Erikson, E., R. L. Cook, G. J. Miller, and R. L. Erikson.** 1981. The same normal cellular protein is phosphorylated after transformation by avian sarcoma viruses with unrelated transforming genes. Mol. Cell. Biol. **1**:43–50.
15. **Fava, R. A., and S. Cohen.** 1984. Isolation of a calcium-dependent 35-kilodalton substrate for the epidermal growth factor receptor/kinase from A-431 cells. J. Biol. Chem. **259**: 2636–2645.
16. **Flower, R. J., and G. J. Blackwell.** 1979. Anti-inflammatory steroids induce biosynthesis of a phospholipase A_2 inhibitor which prevents prostaglandin generation. Nature (London) **278**:456–459.
17. **Flower, R. J., J. N. Wood, and L. Parente.** 1984. Macrocortin and the mechanisms of action of the glucocorticoids. Adv. Inflammation Res **7**:61–69.
18. **Geisow, M. J.** 1986. Common domain structure of Ca^{2+} and lipid-binding proteins. FEBS Lett. **203**:99–103.
19. **Gerke, V., and K. Weber.** 1984. Identity of p36K phosphorylated upon Rous sarcoma virus transformation with a protein purified from brush borders; calcium-dependent binding to non-erythroid spectrin and F-actin. EMBO J. **3**:227–233.
20. **Giugni, T. D., L. C. James, and H. T. Haigler.** 1985. Epidermal growth factor stimulates tyrosine phosphorylation of specific proteins in permeabilized human fibroblasts. J. Biol. Chem. **260**:15081–15090.
21. **Glenney, J. R.** 1986. Two related but distinct forms of the 36,000 Mr tyrosine kinase substrate (calpactins) which interact with phospholipid and actin in a Ca^{2+}-dependent manner. Proc. Natl. Acad. Sci. USA **83**:4258–4262.
22. **Glenney, J. R.** 1986. Phospholipid-dependent Ca^{2+}-binding by the 36-kDa tyrosine kinase substrate (calpactin) and its 33-kDa core. J. Biol. Chem. **261**:7247–7252.
23. **Glenney, J. R., and B. F. Tack.** 1985. Amino-terminal sequence of p36 and associated p10: identification of the site of tyrosine phosphorylation and homology with S-100. Proc. Natl. Acad. Sci. USA **82**:7884–7888.
24. **Glenney, J. R., B. Tack, and M. A. Powell.** 1987. Calpactins: two distinct Ca^{++} regulated phospholipid- and actin-binding proteins isolated from lung and placenta. J. Cell Biol. **104**:503–511.
25. **Gould, K. L., J. A. Cooper, and T. Hunter.** 1984. The 46,000-dalton tyrosine protein kinase substrate is widespread, whereas the 36,000-dalton substrate is only expressed at high levels in certain rodent tissues. J. Cell Biol. **98**:487–497.
26. **Gould, K. L., J. R. Woodgett, C. M. Isacke, and T. Hunter.** 1986. The protein-tyrosine kinase substrate (p36) is a substrate for protein kinase C in vitro and in vivo. Mol. Cell. Biol. **6**:2738–2744.
27. **Greenberg, M. E., and G. M. Edelman.** 1983. The 34 kd $pp60^{src}$ substrate is located at the inner face of the plasma membrane. Cell **33**:767–779.
28. **Haigler, H. T., D. D. Schlaepfer, and W. H. Burgess.** 1987. Characterization of lipocortin I and an immunologically unrelated 33-kDa protein as epidermal growth factor receptor/kinase substrates and phospholipase A_2 inhibitors. J. Biol. Chem. **262**:6921–6930.
29. **Hanks, S. K.** 1987. Homology probing: identification of cDNA clones encoding members of the protein-serine kinase family. Proc. Natl. Acad. Sci. USA **84**:388–392.
30. **Hattori, T., T. Hoffman, and F. Hirata.** 1983. Differentiation of a histiocytic lymphoma cell line by lipomodulin, a phospholipase inhibitory protein. Biochem. Biophys. Res. Commun. **111**:551–559.
31. **Hirata, F.** 1981. The regulation of lipomodulin, a phospholipase inhibitory protein, in rabbit neutrophils by phosphorylation. J. Biol. Chem. **256**:7990–7997.
32. **Hirata, F.** 1984. Roles of lipomodulin: a phospholipase inhibitory protein in immunoregulation. Adv. Inflammation Res. **7**:71–78.
33. **Hirata, F., K. Matsuda, Y. Notsu, T. Hattori, and R. del Carmine.** 1984. Phosphorylation at a tyrosine residue of lipomodulin in mitogen-stimulated murine thymocytes. Proc. Natl. Acad. Sci. USA **81**:4717–4721.
34. **Hirata, F., Y. Notsu, M. Iwata, L. Parente, M. DiRosa, and R. J. Flower.** 1982. Identification of several species of phospholipase inhibitory protein(s) by radioimmunoassay for lipomodulin. Biochem. Biophys. Res. Commun. **109**:223–230.
35. **Hirata, F., E. Schiffmann, K. Venkatasubramanian, D. Salomon, and J. Axelrod.** 1980. A phospholipase A2 inhibitory protein in rabbit neutrophils induced by glucocorticoids. Proc. Natl. Acad. Sci. USA **77**:2533–2536.
36. **Huang, K.-S., P. McGray, R. J. Mattaliano, C. Burne, E. P. Chow, L. K. Sinclair, and R. B. Pepinsky.** 1987. Purification and characterization of proteolytic fragments of lipocortin I that inhibit phospholipase A_2. J. Biol. Chem. **262**:7639–7645.
37. **Huang, K.-S., B. P. Wallner, R. J. Mattaliano, R. Tizard, C. Burne, A. Frey, C. Hession, P. McGray, L. K. Sinclair, E. P. Chow, J. L. Browning, K. L. Ramachandran, J. Tang, J. E. Smart, and R. B. Pepinsky.** 1986. Two human 35 kd inhibitors of phospholipase A_2 are related to substrates of $pp60^{v\text{-}src}$ and of the epidermal growth factor receptor/kinase. Cell **46**:191–199.
38. **Isacke, C. M., C. A. Sauvage, R. Hyman, J. Lesley, R. Schulte, and I. S. Trowbridge.** 1986. Identification and characterization of human Pgp-1 glycoprotein. Immunogenetics. **23**:326–332.
39. **Isacke, C. M., I. S. Trowbridge, and T. Hunter.** 1986. Modulation of p36 phosphorylation in human cells: studies using anti-p36 monoclonal antibodies. Mol. Cell. Biol. **6**:2745–2751.
40. **Karin, M., R. D. Andersen, E. Slater, K. Smith, and H. R. Herschman.** 1980. Metallothionein mRNA induction in HeLa cells in response to zinc or dexamethasone is a primary induction response. Nature (London) **286**:295–297.
41. **Karin, M., and R. I. Richards.** 1982. Human metallothionein genes: molecular cloning and sequence analysis of the mRNA. Nucleic Acids Res. **10**:3165–3173.

42. **Kristensen, T., C. J. M. Saris, T. Hunter, L. J. Hicks, D. J. Noonan, J. R. Glenney, and B. F. Tack.** 1986. Primary sequence of bovine calpactin I heavy chain (p36), a major cellular substrate for retroviral protein-tyrosine kinases: homology with the human phospholipase A_2 inhibitor, lipocortin. Biochemistry **25**:4497–4503.
43. **Lee, S. W., A.-P. Tsou, H. Chan, J. Thoms, K. Petrie, E. M. Eugui, and A. C. Allison.** 1988. Glucocorticoids selectively inhibit the transcription of the interleukin 1β gene and decrease the stability of interleukin 1β mRNA. Proc. Natl. Acad. Sci. USA **85**:1204–1208.
44. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
45. **Mitchell, R. L., C. Henning-Chubb, E. Huberman, and I. M. Verma.** 1986. c-*fos* expression is neither sufficient nor obligatory for differentiation of monomyelocytes to macrophages. Cell **45**:497–504.
46. **Myers, R. F., and M. I. Siegel.** 1984. The appearance of phospholipase activity in the human macrophage-like cell line U937 during dimethyl sulphoxide-induced differentiation. Biochem. Biophys. Res. Commun. **118**:217–224.
47. **Nigg, E. A., J. A. Cooper, and T. Hunter.** 1983. Immunofluorescent localization of a 39,000-dalton substrate of tyrosine protein kinases to the cytoplasmic surface of the plasma membrane. J. Cell Biol. **96**:1601–1609.
48. **Nilsson, K., K. Forsbeck, M. Gidlund, G. Sundstrom, T. Toterman, J. Sallstrom, and P. Venge.** 1981. Surface characteristics of U-937 human histiocytic lymphoma cell line: specific changes during inducible morphologic and functional differentiation in vitro. Hematol. Blood Transfus. **26**:215–221.
48a. **Northup, J. K., K. A. Valentine-Braun, L. K. Johnson, D. L. Severson, and M. D. Holenberg.** 1988. Evaluation of the antiinflammatory and phospholipase-inhibitory activity of calpactin II/lipocortin I. J. Clin. Invest. **82**:1347–1352.
49. **Pepinsky, R. B., L. K. Sinclair, J. L. Browning, R. J. Mattialiano, J. E. Smart, E. P. Chow, T. Falbel, A. Ribolini, J. L. Garwin, and B. P. Wallner.** 1986. Purification and partial sequence analysis of a 37-kDa protein that inhibits phospholipase A_2 activity from rat peritoneal exudates. J. Biol. Chem. **261**:4239–4246.
50. **Powell, M. A., and J. R. Glenney.** 1987. Regulation of calpactin I phospholipid binding by calpactin I light chain binding and phosphorylation by $p60^{v\text{-}src}$. Biochem. J. **247**:321–328.
51. **Radke, K., V. C. Carter, P. Moss, P. Dehazya, M. Schliwa, and G. S. Martin.** 1983. Membrane association of a 36,000-dalton substrate for tyrosine phosphorylation in chicken embryo fibroblasts transformed by avian sarcoma viruses. J. Cell Biol. **97**:1601–1611.
52. **Radke, K., T. Gilmore, and G. S. Martin.** 1980. Transformation by Rous sarcoma virus: a cellular substrate for transformation-specific phosphorylation contains phosphotyrosine. Cell **21**: 821–828.
53. **Radke, K., and G. S. Martin.** 1979. Transformation by Rous sarcoma virus: effects of *src* gene expression on the synthesis and phosphorylation of cellular polypeptides. Proc. Natl. Acad. Sci. USA **76**:5212–5216.
54. **Richards, R. I., A. Heguy, and M. Karin.** 1984. Structural and functional analysis of the human metallothionein-I_A gene: differential induction by metal ions and glucocorticoids. Cell **37**:263–272.
55. **Rovera, G., D. Santoli, and C. Damsky.** 1979. Human promyelocytic leukemia cells in culture differentiate into macrophage-like cells when treated with a phorbol diester. Proc. Natl. Acad. Sci. USA **76**:2779–2783.
56. **Saris, C. J. M., T. Kristensen, P. D'Eustachio, L. J. Hicks, D. J. Noonan, T. Hunter, and B. F. Tack.** 1987. cDNA sequence and tissue distribution of the mRNA for bovine and murine p11, the S-100-related light chain of the protein-tyrosine kinase substrate p36 (calpactin I). J. Biol. Chem. **262**:10663–10671.
57. **Saris, C. J. M., B. F. Tack, T. Kristensen, J. R. Glenney, and T. Hunter.** 1986. The cDNA sequence for the protein-tyrosine kinase substrate p36 (calpactin I heavy chain) reveals a multidomain protein with internal repeats. Cell **46**:210–212.
58. **Sawyer, S. T., and S. Cohen.** 1985. Epidermal growth factor stimulates the phosphorylation of the calcium-dependent 35,000-dalton substrate in intact A-431 cells. J. Biol. Chem. **260**:8233–8236.
59. **Schlaepfer, D. D., and H. T. Haigler.** 1987. Characterization of Ca^{2+}-dependent phospholipid binding and phosphorylation of lipocortin I. J. Biol. Chem. **262**:6931–6937.
60. **Sefton, B. M., K. Beemon, and T. Hunter.** 1978. Comparison of the expression of the *src* gene of Rous sarcoma virus in vitro and in vivo. J. Virol. **28**:957–971.
61. **Sundstrom, C., and K. Nilsson.** 1976. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). Int. J. Cancer **17**:565–577.
62. **Wallner, B. P., R. J. Mattaliano, C. Hession, R. L. Cate, R. Tizard, L. K. Sinclair, C. Foeller, E. P. Chow, J. L. Browning, K. L. Ramachandran, and R. B. Pepinsky.** 1986. Cloning and expression of human lipocortin, a phospholipase A_2 inhibitor with potential anti-inflammatory activity. Nature (London) **320**:77–81.
63. **Zokas, L., and J. R. Glenney.** 1987. The calpactin light chain is tightly linked to the cytoskeletal form of calpactin I: studies using monoclonal antibodies to calpactin subunits. J. Cell Biol. **105**:2111–2121.