Apoptosis of haematopoietic cells can be induced with an antibody against tomoregulin-1

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ABSTRACT We studied tomoregulin-1, a type-I transmembrane protein with two follistatin modules, a unique epidermal growth factor (EGF) domain and a short, highly conserved cytoplasmic tail. Here we report that a number of hematopoietic cell lines (L1210, CEM, Jurkat, U937, K562, JY, THP-1 and T2) express tomoregulin-1 endogenously. In these cells, apoptosis was induced by an antiserum (C29) and purified IgG against the follistatin modules but not by antisera against the EGF-domain or the cytoplasmic tail. Furthermore, in tomoregulin-1, but not in mock, transfected cells C29 induced apoptosis. Apoptosis was monitored through genomic DNA fragmentation, annexin-V staining and caspase-3 activation. Treatment of the cells with C29 in the presence of H89 (a Ser/Thr kinase inhibitor) or 8'-bromo-cyclicAMP revealed that apoptosis was mediated by a cAMP-dependent Ser/Thr kinase. Moreover, C29 increased $[cAMP]_i$ over 5-fold. Together, these data suggest that the C29 antiserum against tomoregulin-1 induces apoptosis of hematopoietic cells.

Keywords:
apoptosis, growth factor, hematopoietic cells, transmembrane signaling, antibodies, EGF, follistatin

INTRODUCTION

During the application of a differential screening approach to *Xenopus laevis* pituitary, several novel cDNAs have been discovered [Holthuis et al., 1995]. One of these cDNAs was designated 7365 and encodes a protein structurally related to transmembrane precursors of the epidermal/transforming growth factor (EGF/TGF-α) family with a unique EGF domain in that all characteristics are present save for an essential Arg residue [Eib and Martens, 1996]. In addition, the 7365 protein contains a signal peptide, two extracellular follistatin (FS) domains, a transmembrane domain and a short cytoplasmic C-terminal region. Two paper described expression patterns of 7365 [Eib et al., 2000; Morais da Silva et al., 2001]. The mouse and human orthologues (GenBank, U19878) were cloned and the name tomoregulin-1 was proposed for this protein [Eib et al., 2000]. A striking degree of amino acid sequence identity between mammalian and amphibian tomoregulin-1 was found, in particular in the 29 amino acid long C-terminal portion of the protein [Morais da Silva et al., 2001]. The short cytoplasmic tail is nearly identical (97%) between mammals and *Xenopus laevis*. In view of the above characteristics we decided to investigate the role of tomoregulin-1 in proliferation or apoptosis of mammalian cell lines. For this study we employed antisera directed against different parts of tomoregulin-1. The use of antisera to unravel signaling pathways has been successful for CD95 and amyloid precursor protein [Krammer, 2000; Yamatsuji et al., 1996]. Following the two papers on expression patterns [Eib et al., 2000; Morais da Silva et al., 2001], we here provide evidence that tomoregulin-1 is involved in Ser/Thr kinase dependent apoptosis of hematopoietic cells.

RESULTS

Structural and genetic information suggested that tomoregulin-1 could play a role in proliferation or apoptosis. For example, the presence of the EGF-like domain and the FS modules that can bind pro-apoptotic activin is reminiscent of a role for tomoregulin-1 in an apoptotic process [Lehto, 2001; Fukuchi et al., 2001; Chen et al., 2000; Lin and Ying 1999]. Also, its localization on human chromosome 9q31 [Eib et al., 1998] represents a region associated with several neuropathies, loss of heterozygosity, deletions and the presence of tumor suppressors [Blumenfeld et al., 1999;Toda et al., 2000; Chadwick et al., 2000; Bolk et al., 2000; Albrecht et al., 1994; Offit et al., 1993; Chaganti et al., 1995; Merlo et al., 1994; Schulttz et al., 1995; Heppel-Parton et al., 1995; Summersgill et al., 1998; Simoneau et al., 1999; Miura et al., 1995; Gailani et al., 1992]. Thus, the possibility that tomoregulin-1 is involved in apoptosis or proliferation was investigated.

We first established by RT-PCR analysis of tomoregulin-1 mRNA that a number of cell lines of hematopoietic origin (L1210, CEM, Jurkat, U937, K562, JY, THP-1 and T2) express tomoregulin-1 endogenously. No expression was observed in adherent carcinoma cell lines: T24 (human bladder carcinoma), CaCo-2 and HT29 (both human colon carcinomas), CMT8 (canine mammary carcinoma) and PC12 (rat pheochromocytoma). All subsequent experiments were performed with the mouse lymphocytic leukemic cell line L1210. We treated L1210 cells with several antisera directed against different parts of tomoregulin-1, namely A59 (raised against the EGF-like domain), B73 (raised against the twelve most C-terminal amino acids of tomoregulin-1) and C29 (raised against a recombinant polypetide corresponding to the FS domains of tomoregulin-1). As a control, no serum was added to the cells or the cells were exposed to pre-immune serum, normal rabbit serum (NRS) or serum against secretogranin-III (a neuroendocrine secretory protein). Neither one of these conditions nor the A59- or B73-treated cells showed a reduction in cell number (Figure 1A). Only C29, the antiserum against the FS domains of tomoregulin-1, clearly reduced cell numbers in a concentration-dependent fashion. A 10^3-fold dilution of C29 in growth medium reduced cell numbers to ~ 50% of untreated L1210 cells. No effect of C29 was

observed when the serum was diluted 10^5-fold or more. The cytotoxic effect could be prevented by the addition of an excess of the recombinant follistatin domain of tomoregulin-1 or by pre-treating C29 with goat-anti-rabbit serum. The effect of 10^3-fold diluted C29 was not restricted to L1210 cells, since similar results were obtained with CEM (64 % survival), Jurkat (54 % survival), U937 (45 % survival), K562 (76 % survival), JY (69 % survival), THP-1 (73 % survival) and T2 cells (74 % survival). No effect of C29 was observed on cells lacking (no PCR product after 40 PCR-cycles) endogenous tomoregulin-1, such as T24, CaCo-2, HT29, CMT8 and PC12 cells (data not shown). Figure 1B shows the effect of C29 on the growth curves of L1210 cells. The growth curves of control cells and C29-treated cells, differing about one doubling time, indicate that C29 does not have an effect on the duration of the cell cycle, but rather induced cell killing. These data also confirm that in the concentrations used, C29 induced a cell death of ~50%. Indeed, we did not observe a C29-mediated effect on the cell cycle distribution (control cells 51% in G1, 41% in S and 8% in G2/M phase; C29-treated cells 48% in G1, 38% in S and 14% in G2M phase). We measured this two days after the addition of C29, therefore (apoptotic) subG1 cells are absent due to complete disintegration of these cells. Furthermore, the parallel growth curves indicated that the cytotoxic effect induced by C29 is initiated soon after the addition of the antiserum and subsequently disappeared, possibly due to a reduced efficacy of the serum. The plateau in the growth curves is caused by the fact that the medium was not refreshed during the time course of this experiment. Whether the fact that the cells are sensitive for C29 only shortly after the addition of C29 is due to breakdown of C29, reduced levels of tomoregulin-1 after C29 addition, the induction of anti-apoptotic factors or tolerance remains to be investigated. The effect of Sephadex-purified C29-IgG is shown in Figure 1C. The fractions with the highest IgG content clearly induced a reduction in cell number, whereas fractions without detectable amounts of IgG did not elicit such an effect. Figure 1C thus clearly indicates that the C29-mediated response is elicited by IgGs rather than an unknown agent. Since C29 recognized a single band of the expected size (~ 41 kDa) in lysates from L1210 cells (Figure 1D) we conclude that C29 is specific for tomoregulin-1. Moreover, following pre-incubation of the antibody with the recombinant antigen, the 41 kDa band disappeared (Figure 1D, lane 4) lysates of tomoregulin-1 negative T24 and Chinese Hamster Ovary cells gave no immuno-reactive product, and no product with a size similar to that of the structurally related FS [Ueno et al., 1987] was observed (Figure 1D, lane 2, and data not shown).

Because we cannot exclude that C29 recognizes an epitope on hematopoietic cells that is different from tomoregulin-1, we transiently transfected tomoregulin-1 deficient CMT8 cells with a construct encoding tomoregulin-1 and measured C29-induced growth inhibition. Interestingly, the tomoregulin-1 transfected cells displayed a growth advantage compared to mock-transfected cells (~ 35% more cells). In mock-transfected cells, C29 had no effect on the growth curves (88 +/- 6 cells/volume without C29 and 83 +/- 7 cells/volume after C29 addition). However, the addition of C29 to cells transfected with tomoregulin-1 led to apoptosis (~ 60% less cells; 119 +/- 17 cells/volume without and 48 +/- 15 cells/volume with C29). These observations indicate that C29 recognizes tomoregulin-1 specifically and that tomoregulin-1 is a survival factor (e.g. a growth factor, a growth factor receptor or a decoy receptor for growth inhibitors).

Cytotoxicity is mediated either by an apoptotic or by a necrotic pathway. In order to discriminate between these two fundamentally different processes, four assays were performed: DNA fragmentation, PARP proteolysis, annexin-V staining and caspase-3 activation. As shown in Figure 2A, C29 induced the fragmentation of genomic DNA isolated from L1210 cells. Already four hours after the addition of the antiserum, the L1210 DNA was cut into oligosomal DNA fragments (lane 3 in Figure 2A), suggestive of the induction of apoptosis. Longer exposure to C29 resulted in even more DNA laddering, comparable to that of cycloheximide-treated L1210 cells (positive control in lane 7, Figure 2A). DNA ladder formation was not restricted to L1210 cells, because similar results were observed in K562 and THP-1cells (data not shown). The second assay we used to establish the process of apoptosis was the staining of cells with annexin-V, measuring the shift of phosphatidylserine (PS) from the inner leaflet of the plasma membrane towards the outer leaflet. A clear increase in the percentage of annexin-V positive cells was observed (Figure 2B). Within three hours of treatment the amount of apoptotic cells increased from 2.5% to 6%, whereas 24 hr of C29 treatment resulted in a significant increase to ~15% annexin-V positive cells. These cells remain negative for propidium iodide (PI) and thus no signs of necrosis were observed during this time period. However, three days after the addition of C29, annexin-V positive cells became also positive for PI, implying that these cells had undergone secondary necrosis. Finally, we measured the effect of C29 on caspase-3 activity. The activity increased from 5.8 +/- 0.1 pmol AMC/min/mg for untreated cells to 7.4 +/- 0.3 pmol AMC/min/mg 4 hr after the addition of C29, while at 24 hr caspase-3 activity doubled to 12.3 +/-

1.2 pmol AMC/min/mg. Western blot analysis revealed a C29-induced cleavage of the 116-kDa poly(ADP-ribose)polymerase (PARP) into a 25-kDa and an 85-kDa fragment [Penning et al., 1994; Lazebnik et al., 1994] and an increase in p53 after the addition of C29 (data not shown). Taken together, the results obtained from the different assays showed that C29 induces apoptosis in L1210 cells.

A region within tomoregulin-1 involved in the apoptotic process may concern its cytoplasmic tail. This tail of 29 amino acids is virtually identical between mammals and *Xenopus laevis* (97% identity, 100% similarity) and is rich in Ser/Thr residues (6 out of 29, 5 of the C-terminal 9 residues). Therefore, we investigated the role of Ser/Thr kinases in C29-mediated apoptosis. The effect of the Ser/Thr kinase inhibitor N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H89) on C29–induced apoptosis is shown in Figure 3A. The inhibition of cAMP-dependent Ser/Thr kinases clearly protected L1210 cells against the apoptotic effect of C29, whereas H89 alone had no effect on the L1210 cells. Protection was already observed with H89 concentrations as low as 0.5 μM, while complete protection was obtained at 10 μM H89. Protection did not occur when H89 was added two days after the addition of C29 (data not shown). This finding indicates that the cytotoxic effect induced by C29 is initiated soon after the addition of the antiserum and thus corroborates the conclusions derived from the growth curves in Figures 1B and the four apoptosis assays. Because we cannot exclude the possibility that H89 had effects in addition to its well-known inhibition of cAMP-dependent Ser/Thr kinases, we measured the effect of drugs that mimic cAMP-dependent processes (Figure 3B). In combination with C29, a strong synergy was observed by 8'-bromo-cAMP (cell-permeable and stable analogue of cAMP). At a concentration of 100 μM 8'-bromo-cAMP, almost complete suppression of viability was observed. The used concentrations of both H89 and 8'-bromo-cAMP are well within the range normally applied [Kiehn et al., 1998; Ali et al., 1998]. Thus, the effects of the inhibition (by H89) and the stimulation (by 8'-bromo-cAMP) of Ser/Thr kinases suggested a role for these kinases in C29-mediated apoptosis. To further substantiate these inhibition/stimulation studies, we measured the effect of C29 on the intracellular cAMP levels. As depicted in Figure 4, a rapid and substantial increase in $[cAMP]_i$ was induced by C29. Already one hour after the addition a significant increase was observed (4-fold), whereas 24 hr after the addition of C29 $[cAMP]_i$ was even further increased up to 10-fold.

DISCUSSION

In this study, we used a number of antisera against different domains of tomoregulin-1 to dissect its function. Only the antiserum directed against an epitope within the follistatin domains induces apoptosis of L1210 cells. For a number of reasons, we conclude that the anti-tomoregulin-1 antibodies in the C29 serum are responsible for the observed effects. *First*, antisera raised against other parts of tomoregulin-1 (the extracellular EGF-like domain and intracellular cytoplasmic tail) as well as antibodies against the unrelated neuroendocrine secretory protein (secretogranin III) were not effective. *Second*, pre-immune serum had no effect on cell numbers, nor did normal rabbit serum. *Third*, pre-treatment of C29 with specific antibodies (goat-anti-rabbit) completely prevented C29-mediated apoptosis. *Fourth*, no effect was observed on cells lacking endogenous tomoregulin-1, such as T24, CaCo-2, HT29, CMT8 and PC12 cells. *Fifth*, excess of the C29 antigen fully abrogated C29-induced apoptosis. *Finally*, Sepharose-A purified C29-IgGs induced a concentration-dependent cell death. The specificity of C29 was shown by transient transfection studies revealing that in tomoregulin-1, but not in mock, transfected CMT8 cells C29 induced apoptosis.

The induction of apoptosis by the C29 antiserum suggests that a modification of the activity of tomoregulin-1 is involved in an apoptotic pathway, but it is unclear whether this is caused by an activation or inactivation of tomoregulin-1. The possibility that C29 blocks tomoregulin-1 activity is more likely than that it increases its activity, based on functional studies with a close relative of tomoregulin-1, named tomoregulin-2 (~50% amino acid sequence identity) [Uchida et al., 1999]. A human brain tomoregulin-2 cDNA was identified by PCR-analysis using degenerate primers corresponding to an EGF-consensus sequence. Tomoregulin-2 was found to weakly tyrosine phosphorylate the erbB-4 receptor suggesting that it may be involved in cell growth, differentiation or apoptosis [Uchida et al., 1999]. Furthermore, tomoregulin-2 has been found to act as a hippocampal and mesencephalic neuronal survival factor, and it may be involved in colorectal polyps and cancer as well as prostate cancer progression [Horie et al., 2000; Young et al., 2001; Glynne-Jones et al., 2001, Mohler et al., 2002]. With respect to the latter, it is interesting to note that tomoregulin-2 is involved in androgen-independent growth of prostate cancer

[Glynne-Jones et al., 2001; Mohler et al., 2002]. In addition, cleavage of tomoregulin-2 in the extracellular domain has been shown [Uchida et al., 1999; Mohler et al., 2002; Lin et al., 2003] and soluble forms of this protein have been found [Uchida et al., 1999]. In view of the structural resemblance between the tomoregulins, tomoregulin-1 may have a physiological function similar to that of tomoregulin-2, thus acting as a growth factor, a growth factor receptor or a decoy receptor for growth inhibiting ligands. This is in line with our findings that tomoregulin-1 is highly expressed in lung tumors (Eib et al, unpublished observations) and in choriocarcinomas *in vitro* (BeWo cell line) and *in vivo* (Penning et al, unpublished observations). Moreover, during urodele limb regeneration and mouse embryonic limb development tomoregulin-1 is upregulated, suggestive of a role in growth factor signaling [Morais da Silva et al., 2001]. Finally, two recent papers indicate an effect of tomoregulin on nodal signaling, again emphasizing a possible role of tomoregulin as a growth factor signaling molecule [Chang et al., 2003; Harms and Chang, 2003]. The other way around, these and our observations therefore suggest that C29 acts by inactivating tomoregulin-1. In view of two recent trials in prostate cancer targeting tomoregulin, it is of the utmost importance to dissect signaling pathways mediated by tomoregulin [Afar et al., 2004; Zhao et al., 2005].

In conclusion, our results together with the recently published data suggest that the novel type I transmembrane protein tomoregulin-1 may function as a growth factor (receptor), whereas inhibition of its function may lead to apoptosis with Ser/Thr dependent kinases in the downstream signaling pathway. Studies with RNAi will be performed to verify this point.

MATERIALS AND METHODS

Cell lines. JY (human EBV-transformed B-cell) and T2 (human/mouse T-cell hybridoma) were cultured in Iscove's medium supplemented with 5% fetal calf serum (FCS). CEM, Jurkat (both human T-cells) and U937 (human histiocytoma) were cultured in 5% FCS supplemented DMEM. K562 (human erythroleukemic cell) and THP-1 (human monocytic cell) were grown in HEPES-buffered RPMI supplemented with 10% FCS and L1210 (mouse lymphocytic leukemic cell) in DMEM with 4.5 g glucose/L and 10% horse serum. All growth media contained antibiotics (penicillin/streptomycin) and cell lines were cultured in a humidified atmosphere containing 5% CO_2 at 37 ^{0}C. L1210 was a gift from Dr. J. Kuiper

(Leiden, The Netherlands). The other cell lines were kindly provided by Dr. C. Figdor (Nijmegen, The Netherlands).

Chemicals. Tissue culture materials were obtained from BioWhittaker Europe (Verviers, Belgium) and Greiner (Alphen aan den Rijn, The Netherlands), H89 (N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide) supplied by Calbiochem (Breda, The Netherlands) and 8'-bromo-cyclicAMP was provided by Sigma (Zwijndrecht, The Netherlands).

Antisera. Antiserum B73 was raised against the twelve most C-terminal amino acids of human tomoregulin-1, with an N-terminal Cys to couple to hemocyanin, which was used as a carrier in the immunization. Antiserum A59, raised against the EGF-like-domain of human tomoregulin-1, has been described previously [Eib and Martens, 1996]. Antiserum C29 was raised against a recombinant polypeptide corresponding to the follistatin domains of human tomoregulin-1 (amino acids 114-218) expressed in *E. coli* as a fusion protein with a hexahistidine tag (Qiagen, Hilden, Germany).

Cytotoxic assays. Cytotoxicity was analyzed by the MTT (adherent cells) or XTT (suspension cells) proliferation assay (Boeringher-Mannheim, Germany) and by means of manual counting on ad random fields in a buckler counting chamber.

Transfection. Transient transfection was performed the Lipofectamine 2000 (Invitrogen, Groningen, the Netherlands) and full-length human tomoregulin-1 cDNA in the pCS2 vector under the regulation of a CMV promoter. Two days after transfection C29 was added (10^3-fold diluted) and two days later cell numbers were analyzed as described above. Transfection experiments were done twice with each in triplicate.

DNA fragmentation. Cells were lysed for 2 hrs at 50 ^{0}C in a nuclear lysis buffer containing 50mM Tris pH8.0, 300 mM NaCl, 5 mM EDTA, 15 mM $MgCl_2$ and 1% NP40. DNA was precipitated with 2 volumes of ethanol, followed by 30 min incubation at –80 ^{0}C, centrifugation (13000 rpm) for 30 min followed by analysis on a 1.7% agarose gel in the presence of EtBr.

Western blotting. Standard procedures were used. Cells were lysed with nuclear lysis buffer as under DNA fragmentation with the addition of protease inhibitors. The polyclonal rabbit antiserum against

poly(ADP-ribose)polymerase (PARP) was kindly provided by Dr. M. VanderCraen (Gent, Belgium), the polyclonal rabbit antiserum against p53 was kindly provided by Dr. G. Boonen (Utrecht, The Netherlands). Enhanced chemo luminescence (ECL) chemicals were derived from Amersham/Pharmacia Biotech, Roosendaal, the Netherlands).

PS staining. To measure PS exposure on FACS, cells were labelled with annexin-V-FITC which specifically binds PS [Van Engeland et al., 1997].

Caspase-3 activity. The activity of caspase-3 was measured fluorometrically by the release of 7-amino-4-methylcoumarin (AMC) from Acetyl-DEVD-7-AMC as described previously [Los et al., 1995].

$[cAMP]_i$. Following lysis of the cells with 3.5% $HClO_4$ and subsequent neutralisation with $KHCO_3$, samples were analysed with a commercially available RIA (Amersham, Uppsala, Sweden) [Penning et al., 1993].

IgG purification. IgGs were isolated from the serum with a Sepharose-A column and elution with Tris.HCl [Sambrook et al., 1989].

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LEGENDS TO FIGURES

Figure 1. The effect of anti-tomoregulin-1 sera on the viability of L1210 mouse lymphocytic leukemic cell. (A) At day zero, cells were seeded in fresh medium supplemented with sera of different dilutions. At day 5, cells were counted as described under Materials and Methods. Antiserum B73 was raised against the twelve most C-terminal amino acids of human tomoregulin-1, with an N-terminal Cys to couple to hemocyanin, the antiserum A59, raised against the EGF-like-domain of human tomoregulin-1, antiserum C29 was raised against a recombinant polypetide corresponding to the follistatin domains of human tomoregulin-1 (amino acids 114-218) expressed in *E. coli* as a fusion protein with a hexahistidine tag. Goat-anti-rabbit (GAR) and the C29-antigen were pre-incubated with C29 1 hr prior to the addition to the cells. Control cells were incubated in normal growth medium (without any further additions), and normal rabbit serum (NRS) and pre-immune serum were added 10^3-fold dilutions). (B) At day zero, cells were seeded in fresh medium supplemented with 10^3-fold dilution of C29. Squares indicate NRS treatment; closed circles represent C29 treatment. Each day, cell numbers were measured as described under Materials and Methods. (C) The effect of purified IgGs on cell viability. Fraction 2 (circles) and fraction 20 (diamonds) contain no protein and serve as controls for the elution buffer; fraction 4 (squares) and fraction 5 (triangles) contain the highest amounts of IgG. The experiments were repeated at least 3 times with 4 independent samples and the standard deviation was less then 10%. (D) Western blotting to measure the specificity of the C29 antiserum. Both the primary antibody C29 and the peroxidase-labelled goat-anti-rabbit antibody (the second antibody) were incubated for three hr at room temperature. Lane 1, lysate of L1210 cells; lane 2, lysate of T24 human bladder carcinoma cells; lane 3, lysate of *E. coli* expressing C29 antigen; lane 4, lysate of L1210 cells, first antibody C29 pre-incubated with C29 antigen. Lane 4 was treated separately from lanes 1-3. Each lane contained a similar amount of protein. The sizes of the marker proteins are 66, 46, 35, 21.5 and 14.2 kDa.

Figure 2. Anti-tomoregulin-1-FS serum (C29) induces apoptosis in L1210 cells. (A) C29 induces DNA fragmentation. Lane 1, 200 bp ladder; lane 2, NRS-treatment no C29; lane 3, C29 treatment for 1 hour; lane 4, C29 treatment for 3 hours; lane 5, C29 treatment for 6 hours; lane 6, C29 treatment for 16 hours; lane 7, C29 treatment for 24 hours; lane 8, cycloheximide treatment for 24 hours. (B) C29 increases the

amount of annexin-V positive cells. Anti sera were diluted 1000-fold. Experiment performed three times, representative experiments are shown.

Figure 3. Ser/Thr kinases are involved in anti-tomoregulin-1-FS serum (C29) mediated apoptosis. (A) The effect of H89, an inhibitor of cAMP-dependent Ser/Thr kinases on C29-induced apoptosis. Squares represent NRS treatment; closed circles represent C29 treatment; triangles represent C29 treatment plus 10 μM H89; open circles represent NRS treatment plus 10 μM H89; crosses represent C29 treatment plus 100 nM H89 (B) The effect of 8'-bromo-cAMP on C29-induced apoptosis. Squares represent NRS treatment; closed circles represent C29 treatment; open circles represent 100 μM 8'-bromo-cAMP; triangles represent C29 treatment plus 100 μM 8'-bromo-cAMP. To allow cellular uptake, drugs were added 1 hour prior to the addition of C29. Cell numbers were measured as described in Materials and Methods, and C29 was diluted 10^3-fold. The experiments were repeated at least 3 times with 4 independent samples and the standard deviation was less then 10%.

Figure 4. Anti-tomoregulin-1-FS serum (C29) increases the amount of intracellular cAMP. Lowest bar, control cells treated for 24 hours with NRS; middle bar, cells treated for 24 hours with NRS and the last hour with 10^3-fold diluted C29; highest bar, cells treated for 24 hours with 10^3-fold diluted C29.

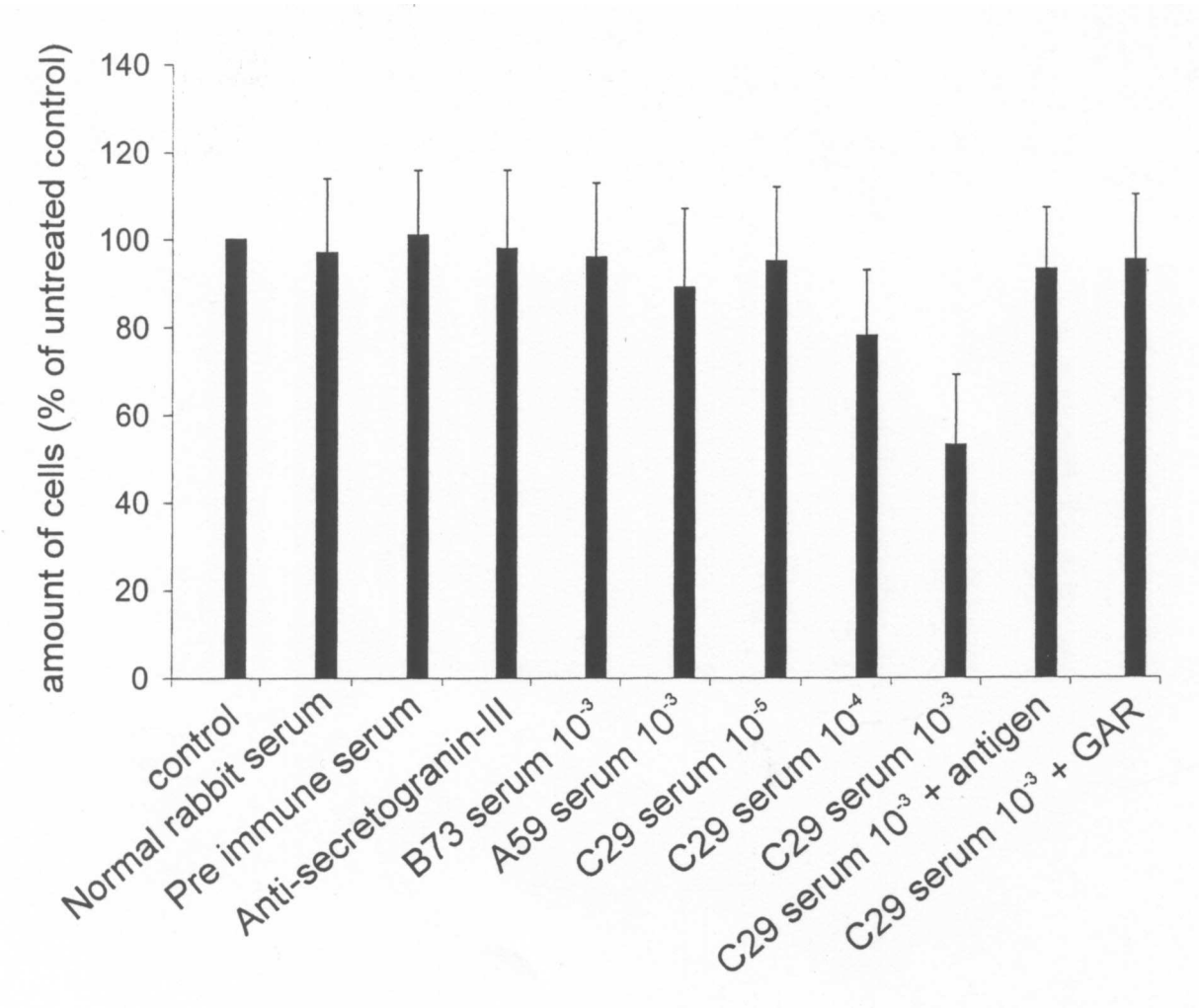

FIGURE 1A. Effects of different antisera on L1210 proliferation.

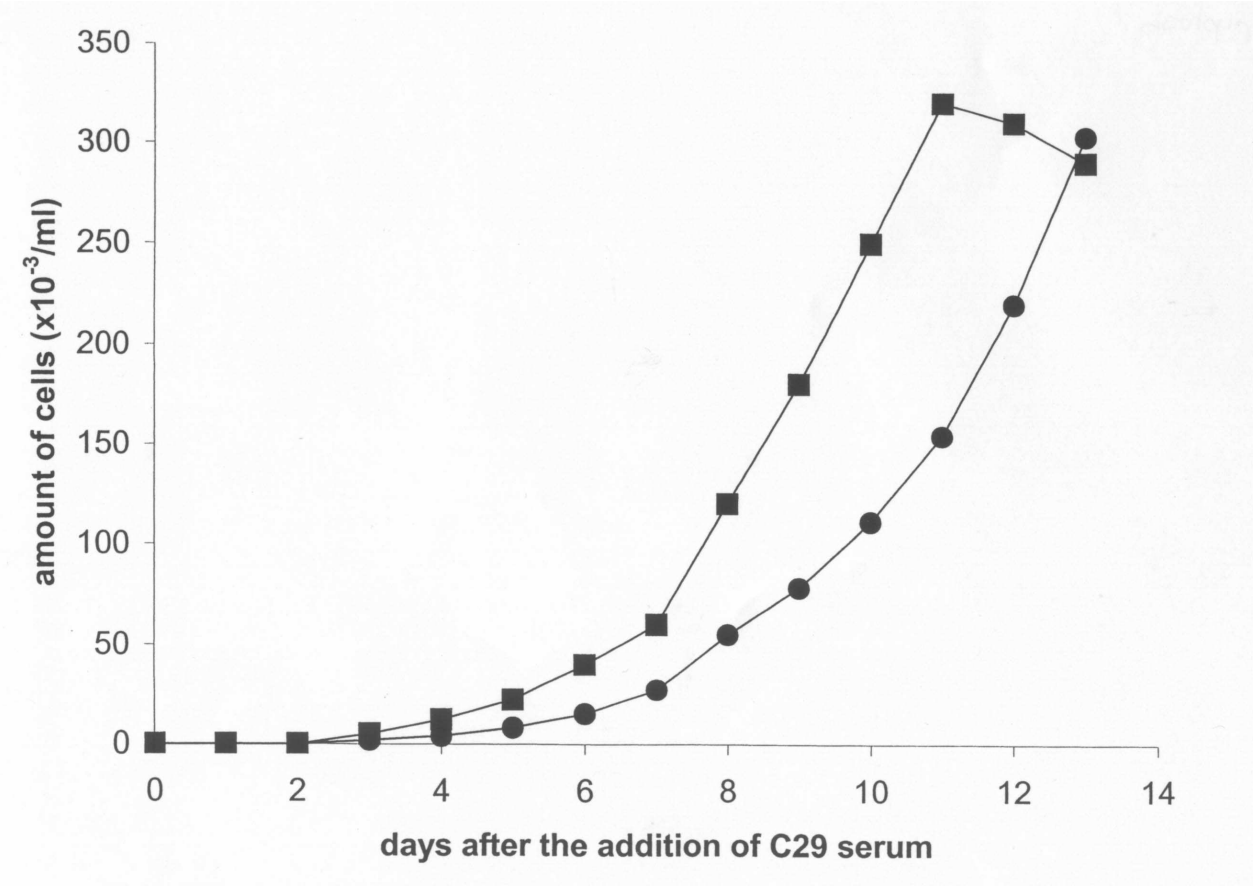

FIGURE 1B. Effect of C29 antiserum on L1210 proliferation

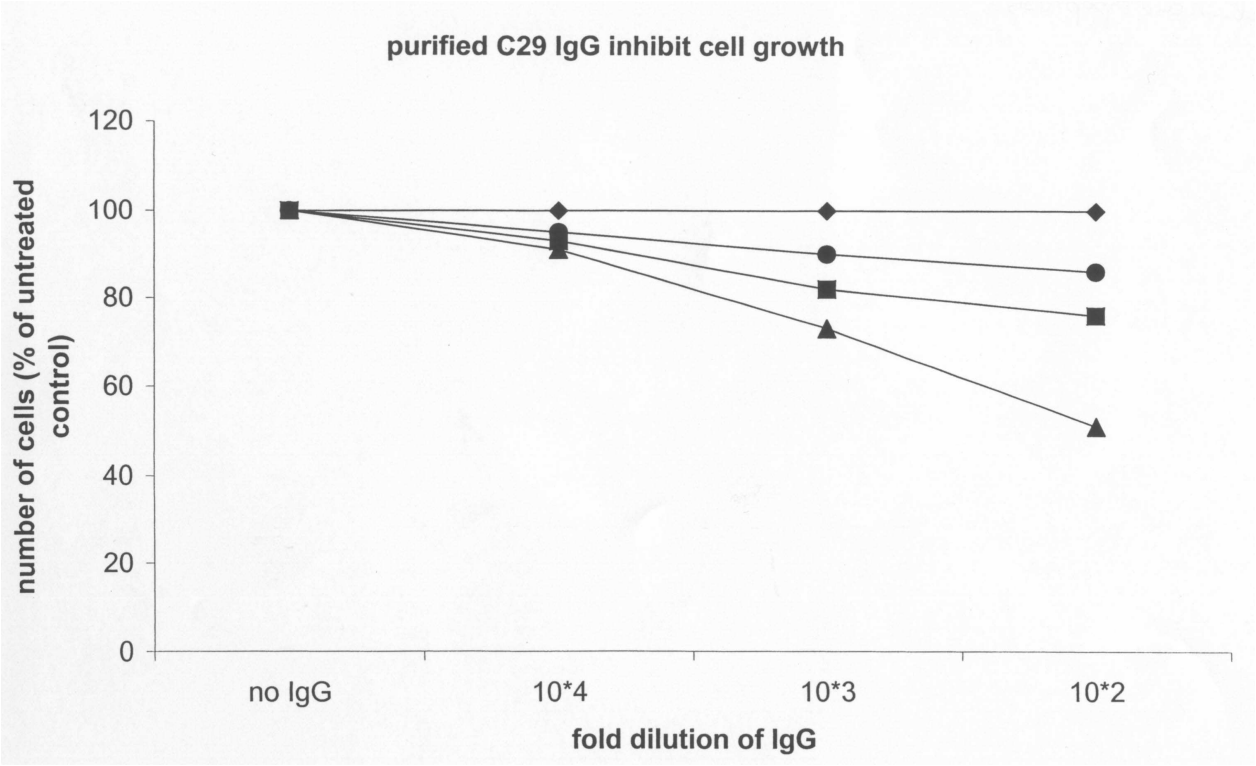

FIGURE 1C. Effect of purified IgGs against the follistatin domain of tomoregulin-1 on L1210 cells.

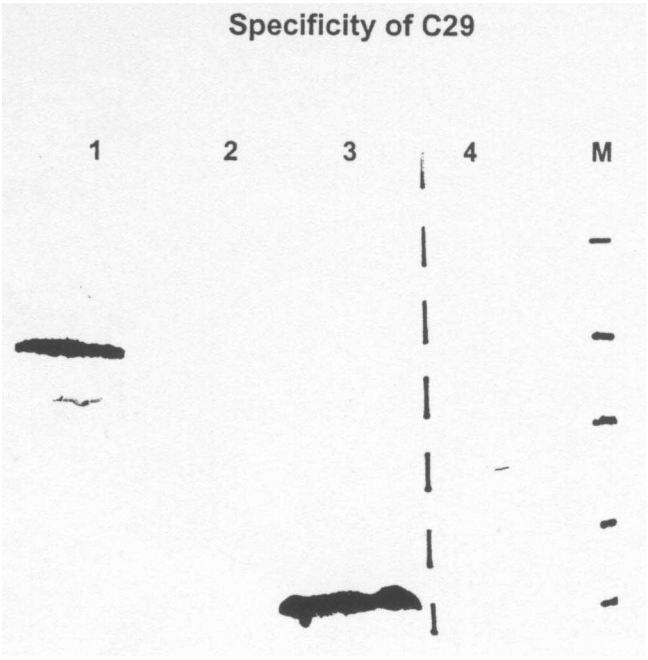

FIGURE 1D. Western blot showing that the C29 antiserum recognizes only tomoregulin-1.

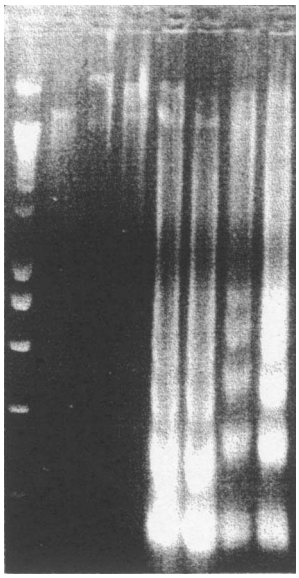

FIGURE 2A. C29 antiserum induces DNA fragmentation in L1210 cells.

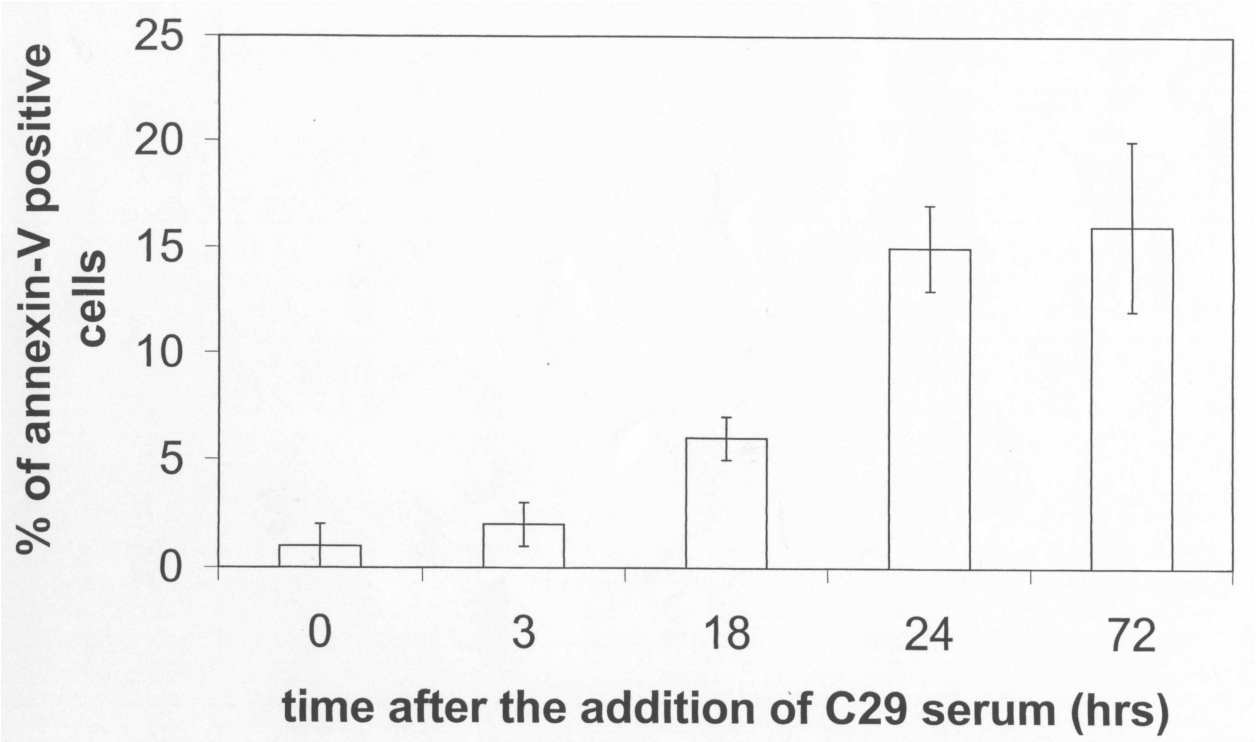

FIGURE 2B. C29 antiserum induces apoptosis as measured with annexin-V in L1210 cells.

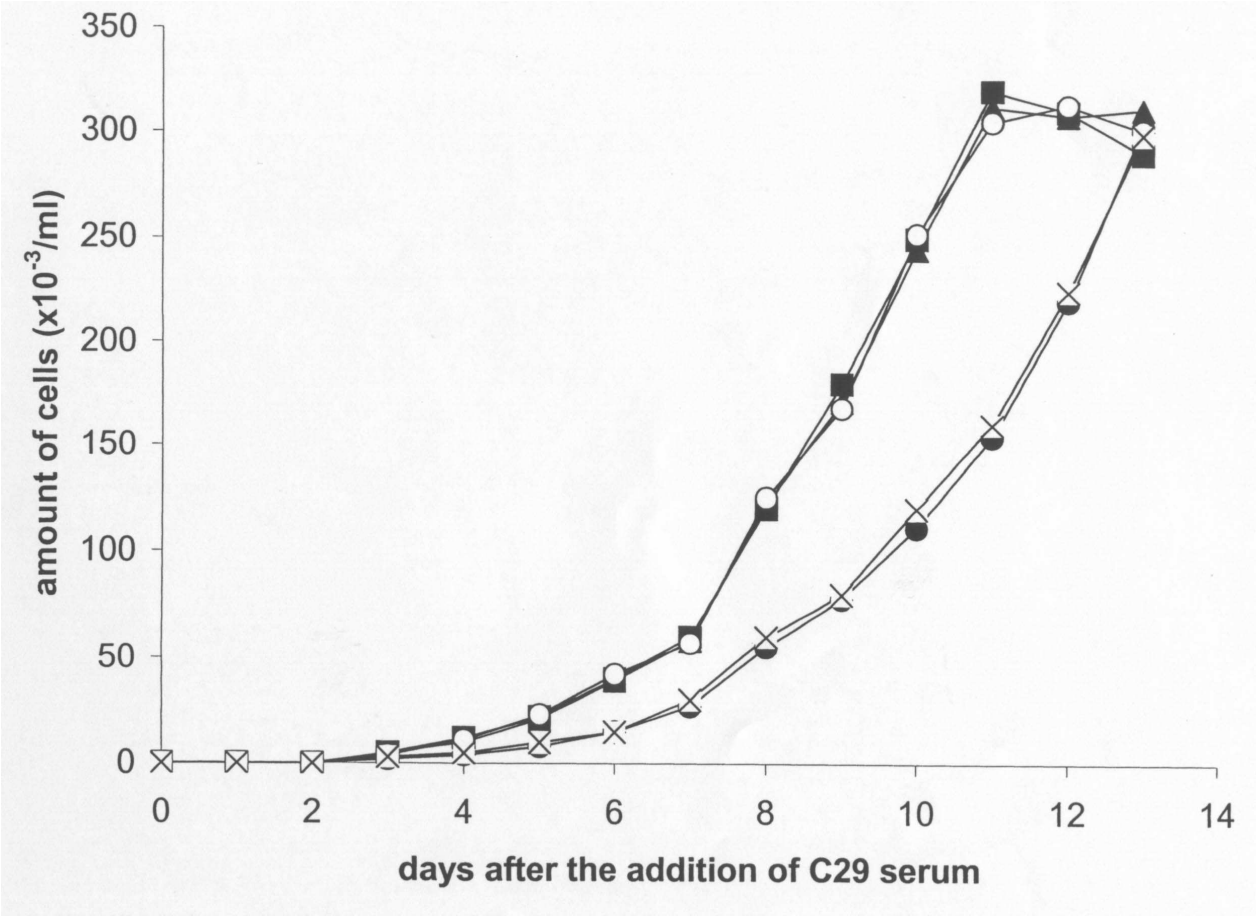

FIGURE 3A. The Ser/Thr kinase inhibitor H89 protects L1210 cells against C29-induced apoptosis.

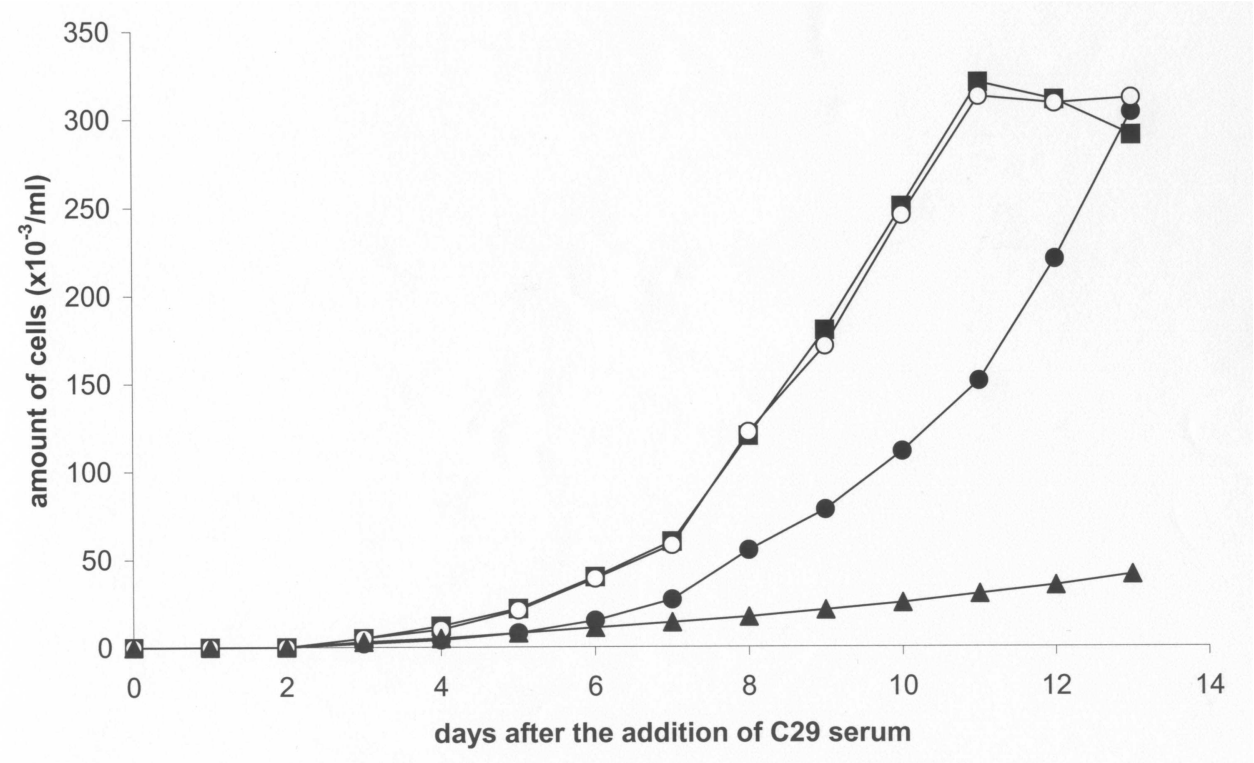

FIGURE 3B. The cAMP analogue 8'-bromo-cAMP strongly stimulates C29-induced apoptosis in L1210 cells.

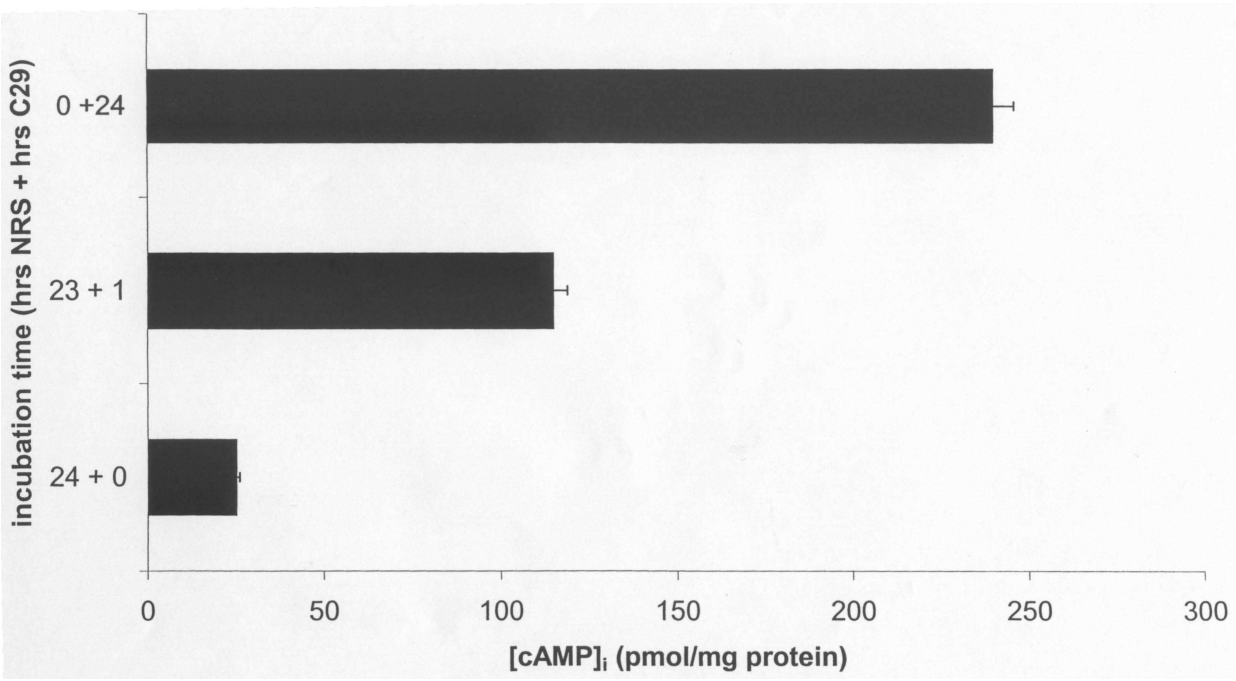

FIGURE 4. Intracellular cAMP levels are induced by C29 treatment in L1210 cells.