Chemically Induced Murine Erythroleukemia Cell Differentiation Is Severely Impaired When cAMP-dependent Protein Kinase Activity Is Repressed by Transfected Genes*

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When exposed to a variety of agents including N,N'-hexamethylene bisacetamide (HMBA)2 and dimethyl sulfoxide (MeSO), Friend virus-infected murine erythroblast leukemia (MEL) cells differentiate into normoblast-like cells that synthesize hemoglobin and other proteins characteristic of mature erythrocytes (for reviews, see Refs. 1 and 2). The events regulating hematopoietic cell differentiation are poorly understood, and the mechanisms of action of agents that induce differentiation in MEL cells remain largely obscure. Numerous studies in several cell systems indicate that cAMP is an important regulator of cell growth and differentiation (for reviews, see Refs. 3-5). In mammalian cells cAMP activates a protein kinase which exists as a tetrameric complex of two regulatory (R) subunits and two catalytic (C) subunits. Enzyme activation occurs when cAMP binds to the R subunits, releasing the C subunits which phosphorylate target proteins (3, 5).

MEL cells contain both type I and type II cAMP-dependent protein kinase (A-kinase) containing R1 and RII subunits, respectively. Exposing MEL cells to MeSO decreases the amount of R1 subunit and increases the amount of R0 and C subunits resulting in an increase in total A-kinase activity (6). A transient increase in the intracellular cAMP concentration occurs 2-8 h after adding chemical inducers to MEL cells, indicating that increased A-kinase activity may be an early regulatory event during MEL cell differentiation (7, 8).

To understand better the role of A-kinase in erythroid cell differentiation, we studied the effects of rendering MEL cells partially or severely deficient in A-kinase activity. To achieve this, we transfected MEL cells with expression vectors encoding cDNAs for either a mutant R1 subunit (Rmut) of A-kinase or the enzyme's specific peptide inhibitor (PKI) (9, 10). Rmut has point mutations in the two cAMP binding sites; its expression in cells represses A-kinase activity and cAMP-controlled events, because holoenzyme containing Rmut is not activated by physiological cAMP concentrations (9, 11). Overexpression of PKI renders cells deficient in A-kinase activity without altering the concentration of R1 protein (10). We found that differentiation induced by HMBA, MeSO, or sodium butyrate was inhibited in the Rmut and PKI transfectants. Inhibition of differentiation was in proportion to the amount of Rmut mRNA and protein expressed. Overexpression of the wild type R1 subunit (Rwt) did not interfere with A-kinase activity or differentiation.

**EXPERIMENTAL PROCEDURES**

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‡Portions of this paper (including "Experimental Procedures," Table 1, A and B, and Fig. 4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
RESULTS

Selection and Screening of Rmut Transfectants

We selected a total of 43 G418-resistant clones in two transfection experiments with either pMT-REVmutneo or pMT-PKI plus pSV2-neo and screened them for expression of either Rmut or PKI, respectively, by measuring A-kinase activity after 48 h of growth in the absence or presence of zinc. Two clones, Rmut/C6.1 and PKI/C15.39, had normal A-kinase activity even when grown in the presence of zinc (Table 1A in the Miniprint) and failed to express Rmut or PKI mRNA, respectively (see below). The remaining 41 clones showed varying degrees of A-kinase deficiency, and growing them in zinc further decreased their A-kinase activity (11 representative clones are shown in Table 1A; as can be seen, many clones showed near-normal A-kinase activity when grown in the absence of zinc and significantly reduced enzyme activity when grown in the presence of zinc). The two most profoundly A-kinase-deficient clones, Rmut/C3 and PKI/C1, displayed <26% and <5% of the enzyme activity of parental cells when grown in the absence and presence of zinc, respectively (Table 1A).

The A-kinase-deficient transfectants grew at a rate less than that of the parental cells with clone Rmut/C3 growing at 1.44 generations per 24 h compared with 1.68 generations per 24 h for the parental cells; moreover, it appeared that the greater the cells’ A-kinase deficiency, the slower they grew. Because we screened the clones as they appeared, we may have inadvertently selected cells with a less severe enzyme deficiency during the initial two transfection experiments. We, therefore, repeated the transfection experiments with pMTREVmutneo and pMTPKI/pSV2-neo and selected clones in media containing G418 plus 500 μM 8-bromo-cAMP and 1 mM theophylline. We reasoned that this double selection would give severely A-kinase-deficient clones a growth advantage over clones expressing the transfected genes less efficiently, since A-kinase-deficient cells are relatively resistant to the growth inhibitory effects of cAMP analogs (9). With this approach we isolated 13 clones, 12 of which were partially A-kinase-deficient when grown in the absence of zinc and were severely enzyme-deficient when grown in the presence of zinc. One clone, Rmut/C9.1, showed normal A-kinase activity in the absence of zinc, but greatly reduced enzyme activity when grown in the presence of zinc (Table 1B; all cells were grown in the absence of 8-bromo-cAMP/theophylline for at least 2 weeks prior to analysis).

Selection and Screening of Rwt Transfectants

After transfection with pMTREVwtneo, G418-resistant clones were screened for overexpression of Rwt by measuring [3H]cAMP binding in cell extracts. We obtained 18 clones in which [3H]cAMP binding was at least 2-3-fold higher than in parental MEL cells when grown in the presence of zinc (six representative clones are shown in Table 1A).

Quantitation of Rmut and Rwt mRNA Expression

When R wt mRNA expression was examined in parental cells, two mRNA species of 1.5 and 3.3 kb were detected (Fig. 1); these two mRNA species have been described previously and appear to be transcribed from the same gene by using different polyadenylation site signals (12, 13). The mRNA transcribed from the transfected Rwt or Rmut expression vectors is about 1.5 kb in size (9).

When the Rmut transfectants shown in Table 1A and 1B were examined for expression of the transfected gene, an excellent correlation was found between Rmut mRNA expression and the degree of A-kinase deficiency (in Fig. 1, clones Rmut/C3 and Rmut/C1 are shown which were selected in G418 or in G418 plus 8-bromo-cAMP/theophylline, respectively). Clone Rmut/C6.1 did not express exogenous Rmut mRNA and showed normal A-kinase activity in the absence and presence of zinc.

When the Rwt transfectants shown in Table 1A were examined, a range of mRNA expression from the transfected gene was found similar to that in the Rmut transfectants with an increase in R wt mRNA expression in the presence of zinc (a representative clone, Rwt/C1, is shown in Fig. 1).

In many of the Rwt and Rmut transfectants, HMBA caused a small but significant increase in mRNA expression from the transfected gene as has been demonstrated by others with a number of different DNA constructs introduced into MEL cells and other cell lines by transient transfection (14). Exposure to HMBA plus zinc induced the highest levels of mRNA expression from the transfected genes in Rwt and Rmut transfectants, higher than the levels induced by zinc alone (Fig. 1).

Quantitation of PKI mRNA Expression

Two low abundance PKI mRNA species of about 4 and 1.5 kb were detected in parental cells (Fig. 2); a low abundance 3.8-kb PKI mRNA has been described previously in mouse tissues (15). In contrast, a 1.2-kb transcript derived from the pMT-PKI construct was easily detected in the transfected clones and increased in response to zinc treatment (in Fig. 2, clone PKI/C1 is shown). The 1.2-kb PKI transcript from the transfected vector was not detected in clone PKI/C15.39 which had normal A-kinase activity unresponsive to zinc treatment (Table 1A).

Quantitation of Rmut and Rwt Protein by Western Blotting

Exposure to HMBA decreased R wt protein in the parental cells by about 80% (Fig. 3), as occurs in MEL cells treated...
tion, since increased catabolism of R1 protein occurs in 

treatment with these two inducing agents (6). HMBA and 

translation or destabilization of R1 protein occurs during 

protein were found in cells treated with both HMBA and zinc 

open bars, 

error 

similar amounts of R1 mRNA from the transfected genes, 

results were obtained with other R1mut transfectants listed in 

Table 1A). 

HMBA- or Me2SO-treated cells, either decreased R1 mRNA 

for 4 days in the absence or presence of 120 μM ZnCl2 and/or 4 mM HMBA. Northern 

blot analysis was performed as described in the legend to Fig. 1, but 

blots were hybridized to a 32P-labeled PK1 cDNA probe as described under “Experimental Procedures.” Equal loading was assured by 

examining ethidium bromide fluorescence of ribosomal RNA bands 

(not shown). The blot was exposed to x-ray film for either 3 h (A) or for 7 days (B). Lane 1, no addition; lane 2, 120 μM ZnCl2; lane 3, 4 mM HMBA; lane 4, 120 μM ZnCl2 plus 4 mM HMBA.

FIG. 2. Northern blot analysis of PKI mRNA expression. Parental MEL cells and MEL cells stably co-transfected with pMT-PKI and pSV2-Neo (clone PKI/C1) were cultured for 4 days in the absence or presence of 120 μM ZnCl2 and/or 4 mM HMBA. Northern 

blot analysis was performed as described in the legend to Fig. 1, but 

blots were hybridized to a 32P-labeled PK1 cDNA probe as described under “Experimental Procedures.” Equal loading was assured by 

examining ethidium bromide fluorescence of ribosomal RNA bands 

(not shown). The blot was exposed to x-ray film for either 3 h (A) or for 7 days (B). Lane 1, no addition; lane 2, 120 μM ZnCl2; lane 3, 4 mM HMBA; lane 4, 120 μM ZnCl2 plus 4 mM HMBA.

FIG. 3. Western blot analysis of R1 protein. Parental MEL 

cells and MEL cells stably transfected with expression vectors for 

R1mut (clones R1mut/C1 and R1mut/C3) or R1wt (R1wt/C1) were 

cultured for 4 days in the absence or presence of 120 μM ZnCl2 and/or 

or 4 mM HMBA. Cells were extracted and 50 μg of cytosolic protein 

were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and examined for R1 protein by Western blotting as described under “Experimental Procedures.” A shows a representative blot. B 

shows the absorbance of the R1 protein band obtained from scanning the autoradiographs of at least three independent Western blots with error bars representing the standard deviation; the relative absorbance was determined as described in the legend to Fig. 1. Lane 1 and open bars, no addition; lane 2 and lined bars, 120 μM ZnCl2; lane 3 and filled bars, 4 mM HMBA; lane 4 and cross-hatched bars, 120 μM 

ZnCl2 plus 4 mM HMBA.

with Me2SO (6). Since R1 mRNA levels are unchanged in 

HMBA- or Me2SO-treated cells, either decreased R1 mRNA 

translation or destabilization of R1 protein occurs during 

treatment with these two inducing agents (6). HMBA and 

Me2SO increase the intracellular cAMP concentration in 

MEL cells which could lead to increased R1 protein degrada-

tion, since physiological cAMP concentrations cannot dissociate the R1mut-C subunit complex (9, 11).

The amount of R1 protein was the same as in parental cells 

in clone R1mut/C6.1 which had normal A-kinase activity (Table 1A) and in PKI transfectants and cells transfected 

with the control plasmid pSV2-Neo; moreover, the amount of 

R1 protein decreased in response to HMBA to the same degree 

in these cells as in parental cells (data not shown).

Quantitation of R1 Protein by 8-N3-[32P]cAMP Labeling

Labeling R1 protein with 8-N3-[32P]cAMP was only applicable 

to the R1wt transfectants, since the affinity of R1mut for cAMP is too low (11). In agreement with the results of the Western blot analysis, clone R1wt/C1 showed 3- and 7-

fold more R1 protein than parental cells in the absence and 

presence of zinc, respectively (Fig. 4 in the Miniprint). HMBA 

treatment decreased the amount of R1 protein in parental cells 

and in clone R1wt/C1 to a similar degree; however, the combination of HMBA and zinc increased the amount of R1 protein in clone R1wt/C1 >10-fold over the amount in HMBA- 

treated parental cells. The amount of R1 protein labeled by 

this procedure was the same in transfected and parental cells 

and did not appear to change with HMBA or zinc. This latter 

finding is in contrast to the results of Schwartz and Rubin (6, 17) who found a significant increase in R1 subunit levels in 

Me2SO-induced MEL cells. However, changes in R1I during 

Me2SO-induced differentiation of MEL cells vary with differ-

ent sublines studied (17), the effect of HMBA on R1I may 

differ, and small (e.g. 2-fold) increases in R1I may not be 

detected by the 8-N3-[32P]cAMP labeling method (6). One 

would not expect a change in R1I protein in the R1wt or R1mut 

transfectants compared with parental cells, since expression of 

R1wt or R1mut protein from a transfected gene does not alter expression of R1I subunits in the transfected cells (9, 12, 18).

A-kinase Activity in HMBA-treated R1mut, PKI, or R1wt 

Transfectants

As reported previously (6), 4 mM HMBA increased A-kinase 

activity in parental cells approximately 2-fold with maximal 

enzyme activity found 48 h after adding HMBA to the culture 

media; enzyme activity was not altered by zinc (Fig. 5). Similar 

results were obtained with two G418-resistant clones which 

had been transfected with the control plasmid pSV2-Neo 

(Table 1A).

Most of the transfectants expressing R1mut or PKI mRNA 

showed some increase in A-kinase activity in response to 

HMBA but enzyme activity remained considerably less than 

that found in HMBA-treated parental cells (Fig. 5). Adding 

zinc to these HMBA-treated transfectants markedly 

decreased A-kinase activity to levels as low as those found when 

the cells were treated with zinc alone. In transfectants 

expressing R1wt mRNA, A-kinase activity was the same or 

slightly higher than that found in parental cells (clone R1wt/ 

C1 in Fig. 5; similar results were obtained with other R1wt 

transfectants listed in Table 1A).

Differentiation of R1mut, PKI, or R1wt Transfectants

Hemoglobin Synthesis—In the absence of HMBA, <1% of 

parental and transfected MEL cells stained positively with 

benzidine, and the hemoglobin content of the cells was <0.1% of 

cellular protein. After exposure to 4 mM HMBA, 75–90%
Role of A-kinase in MEL Cell Differentiation

Fig. 5. A-kinase activity in parental and transfected MEL cells. Parental MEL cells and the stably transfected MEL cells described in the legend to Fig. 1 were cultured in the absence or presence of 120 μM ZnCl₂ and/or 4 mM HMBA. After 48 h, cells were harvested and A-kinase (CAMP-PK) activity was measured as described under “Experimental Procedures.” Results are expressed as percentages of total cellular protein. Results are the mean ± S.D. of at least three independent experiments performed in duplicate. Open bars, no addition; lined bars, 120 μM ZnCl₂; filled bars, 4 mM HMBA; cross-hatched bars, 120 μM ZnCl₂ plus 4 mM HMBA.

Fig. 6. Erythroid differentiation of parental and transfected MEL cells. Parental cells and the stably transfected MEL clones described in the legend to Fig. 1 were examined for hemoglobin production after 5 days of exposure to 4 mM HMBA in the absence (black bars) or presence (cross-hatched bars) of 120 μM ZnCl₂. A, individual cells were scored for hemoglobin production by benzidine staining as described under “Experimental Procedures.” Results are expressed as percent benzidine positive cells after scoring at least three independent experiments performed in duplicate. Viability of all cultures was >85%.

of parental MEL cells or cells transfected with the control plasmid pSV2-Neo stained positively for hemoglobin and spectrophotometric analysis showed that hemoglobin comprised approximately 6% of cellular protein; adding zinc to the media had no significant effect (Table 1A; Fig. 6, A and B). Cells transfected with the RImut or PKI expression vector that were A-kinase-deficient differentiated less than parental cells in response to HMBA (9, 10, 21, 22). Three representative clones, RImut/C1 and PKI/C1 from selection in G418 and RImut/C from selection in G418 plus 8-bromo-cAMP/theophylline, are shown in Fig. 6, A and B. Cells transfected with these two expression vectors that failed to express the transfected genes had normal A-kinase activity and differentiated normally (Table 1A, clones RImut/C6.1 and PKI/C15.39). In all zinc-responsive clones, zinc reduced differentiation to a similar extent as it reduced A-kinase activity (Table 1, A and B; Figs. 5 and 6).

The correlation between the cells' residual A-kinase activity and their ability to differentiate was the same, whether the transfectedants were selected in G418 alone or in G418 plus 8-bromo-cAMP/theophylline (Table 1, A and B). For example, clone RImut/C9.1 was selected in G418 plus 8-bromo-cAMP/theophylline; this clone showed normal A-kinase activity and differentiation when grown in the absence of zinc but reduced enzyme activity and ability to differentiate when grown in the presence of zinc (Table 1B). These results additionally indicate that selection of cells in 8-bromo-cAMP/theophylline per se did not cause inhibition of differentiation.

The Rmut and PKI transfectants were as resistant to Me₂SO- and sodium butyrate-induced differentiation as to the effects of HMBA-induced differentiation (data not shown).

Cells transfected with the Rwt expression vector differentiated normally even in the presence of zinc, and it was clear that large increases in wild type R protein did not interfere with differentiation (Table 1A; clone Rwt/C1 is shown in Figs. 3 and 6).

Commitment and Loss of Proliferative Capacity— Adding zinc up to 48 h after adding HMBA to A-kinase-deficient RImut or PKI transfectants significantly decreased the percent of benzidine positive cells, whereas adding zinc >72 h after HMBA exposure had little effect (data not shown). These results agree with previous findings that 48–72 h of HMBA exposure are required to fully and irreversibly commit MEL cells to differentiate (1, 19).

After exposure to HMBA or HBMA plus zinc, the A-kinase-deficient transfectants showed a growth pattern very similar to that of parental cells; after 5–7 days of culture in the presence of HMBA proliferation ceased, and resuspension in drug-free media did not restore cell growth. These results indicate that the effects of HMBA on cell proliferation and differentiation are dissociated in the A-kinase-deficient cells.

β-Globin mRNA Expression—In addition to hemoglobin synthesis, parental and transfected MEL cells were analyzed for β-globin mRNA by Northern blot analysis (Fig. 7). HMBA increased β-globin mRNA in parental cells about 50-fold over that in untreated cells; there was no significant change in β-globin mRNA when zinc was added to the culture media. In the A-kinase-deficient RImut or PKI transfectants, β-globin mRNA increased less dramatically than in parental cells in response to HMBA and the increase was further inhibited when zinc was added to the media. When a number of the transfectants listed in Table 1, A and B, were examined, there was a good correlation between the cells' residual A-kinase activity and their ability to accumulate β-globin mRNA; Rwt transfectants accumulated β-globin mRNA to the same extent as parental cells (representative clones are shown in Fig. 7).

Since HMBA increases β-globin expression at the transcriptional level (2, 20), these results suggest that A-kinase activity may be required, through an as yet undefined mechanism, for transcriptional activation of the β-globin gene.

DISCUSSION

The expression of transfected cDNA sequences coding for RImut or PKI effectively renders mammalian cells deficient in A-kinase activity and represses cAMP-controlled events (9, 10, 21, 22). We found that expressing these sequences in MEL cells severely inhibited the cells' ability to differentiate.
Our study demonstrated that high levels of Rlwt protein in Rlwt transfectants, as observed in parental cells, but did not lower Rlmut protein levels in the Rlmut transfectants (Fig. 3). Further studies are needed to determine whether the observed differences are due to differences in Rlw t and Rlmut protein degradation. Although HMBA decreased the amount of Rlw t protein in the Rlw t transfectants, combined treatment with HMBA and zinc increased Rl protein to a level >10-fold higher than that found in HMBA-treated parental cells (Fig. 3). In spite of this high amount of Rlw t protein, differentiation proceeded normally as measured by β-globin mRNA expression and hemoglobin production. These results indicate that down-regulation of Rl protein during chemically induced differentiation of MEL cells (6) is not required for normal erythroid differentiation. In contrast, it has been shown by several investigators that down-regulation of the c-myc and c-myc proto-oncogenes is a prerequisite for MEL cell differentiation and that constitutive overexpression of either c-myc or c-myc will inhibit differentiation (25, 26).

A-kinase deficiency does not appear to globally interfere with the cells’ ability to differentiate, since we recently found that the A-kinase-deficient transfectants differentiate normally in response to hemin. Unlike HMBA and Me2SO, hemin does not activate globin gene transcription in MEL cells, but instead seems to regulate globin chain synthesis at the post-transcriptional level (20, 27). Thus, it appears that the A-kinase-deficient MEL cells have the capacity for normal globin mRNA translation in the presence of hemin. Studies are underway to determine whether HMBA-induced transcriptional activation of globin and other erythroid-specific genes is impaired in the A-kinase-deficient MEL cells.

The role of A-kinase in differentiation appears to vary between cells of different lineages, since nerve growth factor-induced differentiation of PC 12 cells is not impaired in Rlmut transfectants (21). It should be noted, however, that an important role of cAMP has been demonstrated in numerous studies of mammalian and lower eukaryotic cell differentiation (3–5, 28). The transfected MEL cells described in this study provide evidence for an important role of A-kinase in erythroid cell differentiation. Furthermore, they offer the opportunity to study the regulatory proteins phosphorylated by A-kinase during MEL cell differentiation and to determine whether the enzyme is directly or indirectly involved in activating erythroid-specific gene transcription.

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REFERENCES
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EXPERIMENTAL PROCEDURES

Fibroblasts: IL-6, 8, 11, 10, 12, 13, 14, 15, 16, 17, 18, and 22. Cells were grown in 10 cm diameter tissue culture dishes. Growth media was replaced daily. Following 10 days in serum-free media, the medium was replaced with medium containing 200 μM IL-6 and 10 μM cytosine arabinoside. The cells were subcultured with 0.1% trypsin-EDTA for analysis. A-kinase activity was assayed by the method of Jahnzen et al. (1989). The cells were lysed in buffer containing 10% glycerol, 50 mM HEPES, pH 7.4, 1 mM DTT, and 10 μM EGTA. Cell lysates were centrifuged at 13,000 g for 15 min at 4°C. The supernatant was used for analysis. A-kinase activity was assessed by assaying for protein kinase activity as described.

Cells were grown and assayed as described above except the extraction density was 10 x 10^6 cells/mL. IL-6 binding was assessed by sodium sulfite precipitation (30).

Cells were cultured in 10 cm diameter tissue culture dishes. The cells were subcultured with 0.1% Trypsin-EDTA for analysis. A-kinase activity was assessed by the method of Jahnzen et al. (1989).

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### Role of A-kinase in MEL Cell Differentiation

#### TABLE 1A

**A-kinase Activity and Hemoglobin Differentiation in Transfected MEL Cells Selected in a.s.**

MEL cells were stably transfected with expression vectors for Rnmt, PKI, Rnwt or the control plasmid pV7-neo and selected in 6-51 as described in Experimental Procedures. A-kinase activity was measured after 2 d of culture in the absence or presence of 120 µM ZnP, as described in Experimental Procedures; enzyme activity is expressed as a percent of the control. Cytosolic protein was measured as described in Experimental Procedures. Hemoglobin production was assessed by benzidine staining, as described in Experimental Procedures. After 5 d of culture in 6 µM MMA in the absence or presence of 120 µM ZnP, Rnwt protein in the Rnwt transfectants was measured by Western blotting (33) and was at least 2.5-fold higher than parental MEL cells.

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#### TABLE 1B

**A-kinase Activity and Hemoglobin Differentiation in Transfected MEL Cells selected in a.s. plus 8-Bromo-cAMP/Theophylline**

MEL cells were transfected with expression vectors for Rnwt or PKI as described in Experimental Procedures and selected for growth in media containing 8-bromo-cAMP (80 µM) and theophylline (10 µM). Kinase activity and hemoglobin accumulation were measured as described in the legend to Table 1A.

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<td>PKI/C16.3</td>
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#### Fig. 4

Photoaffinity labeling of Rα subunits in cytosolic extracts. Parental MEL cells and MEL cells stably transfected with the Rnwt expression vector (clone Rnwt/C1) were cultured for 4 d in the absence or presence of 120 µM ZnP, and/or 4 µM MMA. Cell extracts were prepared and 10 µg of cytosolic protein was labeled with 8-N-p-Tyr-cAMP as described in Experimental Procedures. Protein bands migrating at 80,000 and 90,000 Da are Rα subunits. Proteins with apparent molecular masses of 29 and 52 kDa, respectively, whose labeling was abolished in the presence of 0.01 µM cAMP (not shown). Lane 1: no addition; lane 2: 120 µM ZnP; lane 3: 4 µM MMA; lane 4: 120 µM ZnP, plus 4 µM MMA.