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Subtype-specific increase in G-protein α-subunit mRNA by interleukin 1β

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The guanine nucleotide regulatory proteins (G-proteins) which are substrates for ADP-ribosylation by pertussis toxin (α_{i-1}, α_{i-2}, α_{i-3} and α_o) transduce a variety of hormonal signals. Endothelial cells express mRNA for three α_i subtypes although the level of α_{i-1} mRNA is very low. Interleukin 1β (IL1β), a pleiotropic inflammatory mediator which stimulates a complex series of responses in human endothelial cells leading to increased coagulation and platelet adhesion, increases expression of one subtype of α_i (α_{i-2}) mRNA in human endothelial cells as determined by Northern blot analysis without affecting the level of mRNA for other α-subunits. These studies show that mRNA levels for α_i subtypes are independently regulated, suggesting that there may be subtype specificity in the cell's requirements for the G_i class of signal-transducing proteins.

Interleukin 1; Guanine nucleotide regulatory protein; mRNA; (Endothelial cell)

1. INTRODUCTION

G-proteins are heterotrimers comprised of α-, β- and γ-subunits and are distinguished by variations in α-subunits (reviews [1,2]). The α-subunits of G_s (α_s) which mediate stimulation of adenylate cyclase and regulate ion channels can be covalently modified by cholera toxin-catalyzed ADP-ribosylation. Another family of G-protein α-subunits are substrates for covalent modification by pertussis toxin. Two members of this family are expressed only in the retina. In other cells, members of this family are involved in hormonal regulation of inositol phospholipid metabolism, arachidonic acid release, and ion channels. Several cDNAs have been identified for α proteins in this group that each contain a putative site for ADP-ribosylation by pertussis toxin [1,2]. The proteins encoded by these cDNAs have been called α_{i-1}, α_{i-2}, α_{i-3} and α_o. In addition, another type of α-subunit, called α_z, has recently been identified which is different from α_s, but also lacks the site for ADP-ribosylation by pertussis toxin [3,4]. The function of this G-protein α-subunit is not yet known.

The three forms of α_i are extremely similar to each other (human α_{i-1} is 94% identical to α_{i-3} and 85% identical to α_{i-2}). It is not yet known whether they have independent functions or whether the cell can use any member of the group interchangeably. Reconstitution experiments argue for a great deal of cross-talk among G-protein α-subunits [1]. On the other hand, the strict conservation of α_i subtype sequences across species suggests that the subtle differences in the isoforms are, in fact, important.

Interleukin 1β (IL1) is a pleiotropic inflammatory mediator that exerts diverse effects upon lymphocytes and many other cell types [5], including cultured human endothelial cells (HEC) [6]. IL1β effects on HEC apparently require DNA

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and protein synthesis; however, little else is known about intracellular events underlying IL1β target cell responses. G-proteins appear to transduce signals important in endothelial growth and inositolphospholipid metabolism (in preparation). Because IL1β regulates many endothelial cell functions, including responses initiated at the plasma membrane [6–10], we postulated that IL1β may alter expression of G proteins which transduce a variety of transmembrane signals. We now show that activation of the IL1β receptor in endothelial cells can lead to changes in the level of mRNA for one α_i subtype, but not for others. Independent regulation of α_i subtype mRNA provides another line of evidence that there may be separate roles for the α_i subtypes.

2. MATERIALS AND METHODS

2.1. *Cell culture*

Human umbilical vein endothelial cells from 2 –5 cords were isolated and grown as described [6]. The cultures were 100% endothelial cells as judged from DiI-Ac-LDL uptake; DiI-Ac-LDL, acetylated low density lipoprotein reacted with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate [11].

2.2. *RNA analysis*

Total cellular RNA was prepared by the guanidine isothiocyanate/cesium chloride method [12]. Northern analysis was performed using ^{32}P-labeled probes described below. Filters were washed twice at 52°C (bovine or rat probes) or 65°C (human probes) in 0.1% SDS and 0.2 × SSC (1 × SSC = 0.15 M NaCl/15 mM sodium citrate, pH 7.0). To quantitate the amount of RNA loaded on agarose gels, a 15-fold molar excess of a ^{32}P-labeled oligonucleotide probe (5′-ACGGTATCTGATCGTCTTCGAACC-3′) complementary to rat 18 S ribosomal RNA [13] was hybridized to filters. For each experimental observation , at least two Northern blots were performed with 18 S standardizations. RNA sizes were determined by comparison to synthetic RNA markers (Bethesda Research Laboratories, Bethesda, MD). Autoradiograms were scanned using an LKB 2222-010 Ultroscan XL laser densitometer. The absorbance of bands was expressed as a multiple of the control. The one-sample *t*-test was used to compare experimental data. Data are expressed as means ± SD.

2.3. *Probes*

Because of the high degree of conservation of the G-protein α-subunits rat probes for $\alpha_{i\text{-}2}$, α_o and α_s react specifically with their counterparts in human mRNA. Initial experiments were performed with the rat probe for $\alpha_{i\text{-}2}$, α_s and α_o kindly provided by Dr R. Reed (Johns Hopkins University, Baltimore, MD) and partial bovine cDNA clone for $\alpha_{i\text{-}1}$ and $\alpha_{i\text{-}2}$ (α_h) which we had previously reported [14]. For rat cDNAs the *Eco*RI insert which contained the full coding sequence was used to probe Northern blots. For bovine $\alpha_{i\text{-}1}$ we used the *Sma*I-*Xba* fragment described by Michel et al. [14]. Subsequently, this laboratory isolated cDNA clones for human $\alpha_{i\text{-}1}$, $\alpha_{i\text{-}2}$ and $\alpha_{i\text{-}3}$. Selected experiments were repeated with the ^{32}P-labeled human cDNA probes to verify the conclusions reached with heterologous probes as well as to extend the studies to human $\alpha_{i\text{-}3}$. The cDNA fragments used as probes were the same as those detailed by Kim et al. [15].

2.4. *ADP-ribosylation*

ADP-ribosylation of lysed endothelial cells by pertussis toxin was performed as described, except that the reaction mixture also contained 0.1% Lubrol Px (Lubrol 12A9, ICI) and 0.015% bovine serum albumin [16]. Two-dimensional gel electrophoresis was carried out as described by O'Farrell et al. [17].

3. RESULTS AND DISCUSSION

3.1. *IL1β increases $\alpha_{i\text{-}2}$ mRNA in HEC*

We have previously shown that human umbilical vein endothelial cells express easily measurable mRNA for $\alpha_{i\text{-}2}$ and $\alpha_{1\text{-}3}$, but very low amounts of $\alpha_{i\text{-}1}$ mRNA [15]. A rat cDNA for $\alpha_{i\text{-}2}$ recognized a prominent 2.5 kb species and a much less prominent 3.1 kb species in mRNA from HEC cells (fig.1). After treatment of HEC with recombinant IL1β (Cistron Biotechnology, Pine Brook, NJ) for 24, 48 or 72 h, the quantity of predominant 2.5 kb mRNA (measured by densitometry of autoradiograms) increased 1.6 ± 0.6, 2.6 ± 0.4, and 3.5 ± 1.0 fold, respectively ($n = 4$, $p < 0.01$). The amount of 18 S RNA in each lane was quantitated with an oligonucleotide probe (see section 2). This measurement was used as a control to monitor the amount of RNA loaded in each lane. There was no increase in hybridization of a ^{32}P-labeled mouse β-actin cDNA in HEC treated with IL1β (not shown). The IL1β-induced increase in $\alpha_{i\text{-}2}$ mRNA was dose-dependent, with peak response at 5 U/ml (fig.2). When human cDNA probes became available, we reanalyzed the filters with human $\alpha_{i\text{-}2}$ cDNA and obtained identical results to those with the rat $\alpha_{i\text{-}2}$ cDNA. The increase in mRNA level was specific to the $\alpha_{i\text{-}2}$ subtypes, since we found no increase in $\alpha_{i\text{-}3}$ mRNA (fig.2) using human $\alpha_{i\text{-}3}$ cDNA probes. There was also no increase in the very low level of $\alpha_{i\text{-}1}$ mRNA. The same filters were also analyzed for $\alpha_{i\text{-}2}$ and $\alpha_{i\text{-}3}$ mRNA and found to contain the expected levels of mRNA for these subtypes. Therefore, the very small amount of $\alpha_{i\text{-}1}$ mRNA detected was not due to degradation of mRNA or loading of insufficient mRNA. HEC RNA hybridized to rat α_s cDNA revealed a 2.0 kb RNA species; however, no consistent increase was observed after IL1β treatment. The rat α_o cDNA

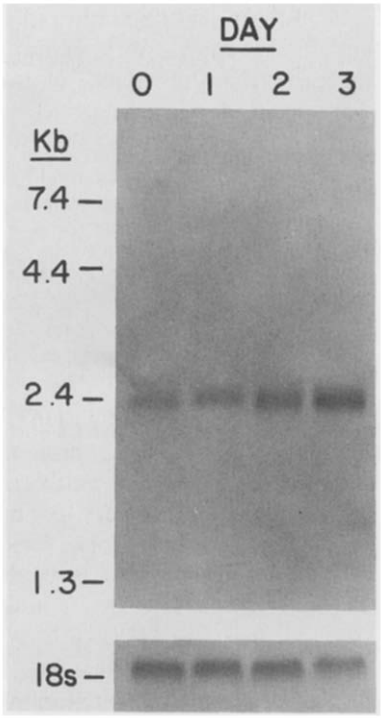

Fig.1. IL1β induces an increase in α_i-like mRNA in cultured human endothelial cells. HEC were incubated with 5 U/ml of IL1β for 0, 1, 2, or 3 days. For this Northern analysis, 10 μg total cellular RNA was loaded on each lane. Hybridization to rat α_i cDNA revealed a 2.5 kb RNA species and a 3.1 kb species (poorly visible on this exposure). Below, the hybridization of an oligonucleotide complementary to 18 S ribosomal RNA shows that equal amounts of RNA were loaded. This Northern blot is representative of 4 similar experiments.

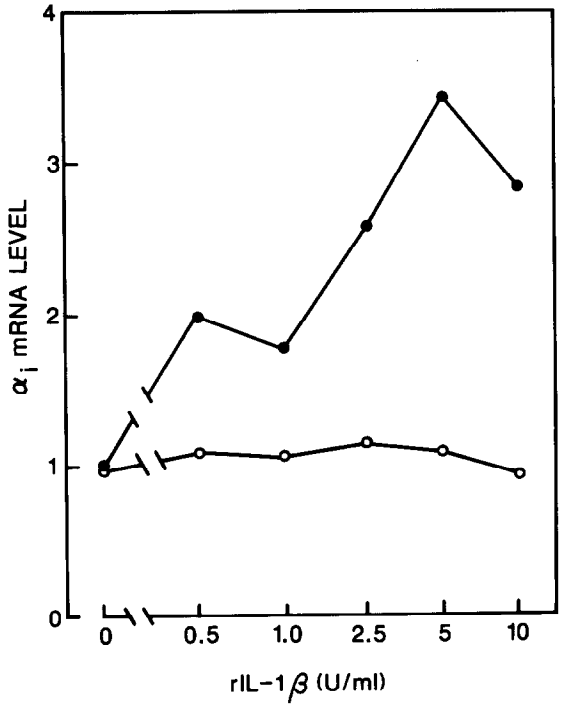

Fig.2. Dose response of α_{i-2} mRNA in HEC treated with IL1β. Cells were treated with varying IL1β concentrations for 3 days and the total cellular RNA was analyzed as in fig.2. Data are expressed as the ratio of the absorbance of the 2.5 kb α_{i-2} mRNA to that of the 18 S RNA. This experiment is representative of 3 similar experiments: (●) α_{i-2} (results are shown with rat α_{i-2}; similar results were obtained with human α_{i-2}); (○) α_{i-3} (human α_{i-3} probe).

failed to recognize mRNA species in HEC RNA despite the use of enhancing screens and prolonged autoradiogram exposures.

Vascular endothelial cells respond to inflammatory mediators with a variety of effects. Some of these effects, such as leukocyte adhesion and platelet-activating factor production, occur during the first hours of treatment [6]. However, other effects such as prostacyclin synthesis [10] and increased plasminogen activator inhibitory activity [7], require longer incubation periods. IL1β specifically increases the levels α_{i-2} mRNA with a dose-response pattern which closely parallels the induction of procoagulant and regulation of the fibrinolytic system [8]. The increase in α_{i-2} mRNA in HEC in response to IL1β occurs gradually over the first 3 days of exposure, suggesting that G-protein regulation is not involved in the generation of early procoagulant activity but may play a role in the later events of the inflammatory response.

3.2. *HEC contain multiple substrates for ADP-ribosylation by pertussis toxin*

Fig.3A shows the pattern of ADP-ribosylated α-subunits in control and IL1β-treated HEC. On one-dimensional gel electrophoresis, we were unable to detect any change in amount of [^{32}P]ADP-ribose incorporated into the pertussis toxin substrates. Addition of exogenous brain $\beta\gamma$-subunit did not alter the pattern (not shown). Although endothelial cells contain multiple kinds of α_i-subunits, we could not detect more than one band on a one-dimensional SDS PAGE.

Two-dimensional gel electrophoresis does reveal the heterogeneity of α_i-subunits in endothelial cells (fig.3). The major ADP-ribosylated α-subunit of endothelial cells migrates similarly to ADP-

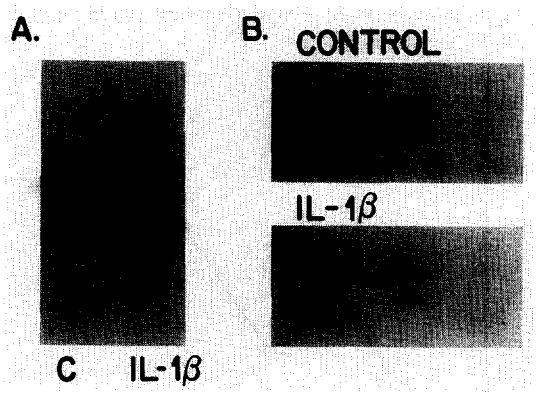

Fig.3. Endothelial cells were incubated for 3 days with or without 5 U/ml IL1β. Cells were harvested, lysed and ADP-ribosylated as described in section 2. (A) Autoradiogram of a one-dimensional SDS-PAGE analysis of control and treated cells. Both lanes were loaded with 44 μg protein. (B) Two-dimensional gel analysis of samples shown in A. On two-dimensional analysis we sometimes saw a radioactive approx. 35 kDa spot below the major pertussis toxin substrate. The spot was very faintly present on a one-dimensional gel. We do not know what this spot represents but there was no change in this spot in IL1β-treated cells.

ribosylated α_{i-2} purified from bovine brain (not shown), and probably represents endothelial cell α_{i-2}. Two-dimensional gels revealed no change in the amount of any ADP-ribosylated protein after interleukin 1β treatment. Antibodies would allow a more definitive assignment of subtype, but we were unable to obtain sufficiently strong signals from anti-peptide antibodies for reliable identification or quantitation.

An increase in an α_{i-2} protein might not be detectable by the methods we used because it might be considerably smaller than the increase in mRNA level. Since α_{i-2} is the most abundant isoform in the unstimulated HEC, small changes would be difficult to document. Alternatively, the newly formed protein might not be a substrate for ADP-ribosylation by pertussis toxin. The interaction of pertussis toxin with the G-protein subunits is complex and may not reflect changes in the amount of a specific α-subunit. The development of more sensitive immunological methods will be needed to allow us to measure possible changes in G-protein levels.

These studies demonstrate that mRNA levels for the subtypes of the α_i-like proteins are independently regulated. We do not know whether regulation is at the level of transcription or mRNA stability. The independent regulation of α_i subtype mRNA suggests that the α_i subtypes may not be interchangeable, but that the cell may require specific subtypes for specific functions.

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