Interleukin 2 and a lactogen regulate proliferation and protein phosphorylation in Nb2 cells

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Cell proliferation and protein phosphorylation in response to activation of lactogenic and interleukin 2 (IL-2) receptors were studied in Nb2 cells, a rat T-lymphocyte cell line. Human growth hormone (hGH) and rat IL-2 stimulated Nb2-cell proliferation to approximately the same degree, and the actions of both mitogens were potentiated by phorbol 12-myristate 13-acetate (PMA). A monoclonal antibody specific for the rat IL-2 receptor inhibited the mitogenic actions of rat IL-2, but not those of hGH. Exposure of Nb2 cells to either mitogen for 2–3 h increased phosphorylation of an 18600-Da protein and decreased phosphorylation of a 15600-Da protein. PMA also inhibited phosphorylation of the latter protein, but, by itself, PMA did not stimulate phosphorylation of the 18600-Da protein. Overall, the results suggest that hGH and IL-2 act through separate receptors to stimulate proliferation of Nb2 cells, and that some of the actions of both mitogens may be mediated, in part, through regulation of protein phosphorylation.

INTRODUCTION

Interleukin 2 (IL-2), a lymphokine secreted by T cells, is generally considered to be one of the major regulators of T-cell function and proliferation (for review see [1]). It is increasingly evident, however, that lactogenic hormones also play an important role in regulation of immune function. Suppression of circulating concentrations of prolactin decreases immune function, and immunocompetence can be restored by the administration of lactogenic hormones [2,3]. Furthermore, other studies [4–7] have suggested that prolactin maintains T-cell immunocompetence and that the immunosuppressive effects of cyclosporin may be mediated, in part, through inhibition of prolactin binding. Receptors for prolactin have been identified on T and B lymphocytes [5,8], including a rat T-cell line (Nb2 cells; [9]) that proliferates in response to lactogens [8]. Activation of lactogen receptors in Nb2 cells increases ornithine decarboxylase activity [10–12] and the amount of mRNA coding for the proto-oncogene *c-myc* [13]. Studies [14,15] have shown that IL-2 also increases *c-myc* mRNA in T cells; thus some actions of prolactin and IL-2 in T cells may share a common mechanism. One of the initial actions of IL-2 may be activation of protein kinase C, because IL-2 stimulates translocation of this enzyme in T cells [16], and phorbol esters, which are potent activators of kinase C [17], mimic the effects of IL-2 on inhibition of adenylate cyclase activity [18], production of interferon [19], stimulation of cell proliferation [20] and phosphorylation [21,22]. The mitogenic actions of prolactin in Nb2 cells also may involve kinase C, because an inhibitor of this enzyme decreases prolactin-induced thymidine uptake [23]. Moreover, it has been shown that phorbol

esters potentiate the mitogenic actions of lactogens [24] in Nb2 cells. In this study, we have examined the roles of a lactogen, IL-2 and phorbol 12-myristate 13-acetate (PMA) in regulating proliferation and protein phosphorylation in Nb2 cells. The lactogen used in this study [human growth hormone (hGH)] also has somatotrophic properties; however, results of previous studies [8,25] have shown that the somatotrophic actions of this hormone do not affect Nb2-cell proliferation.

MATERIALS AND METHODS

Materials

hGH (79-7-23H, 2.2 i.u./mg) was a gift from Dr. H. G. Friesen (Winnipeg, Canada), rat IL-2 was purchased from Collaborative Research Inc. (Lexington, MA, U.S.A.) and mouse IL-2, produced by recombinant technologies, was purchased from Genzyme (Boston, MA, U.S.A.). The purity of the rat preparation has not been assessed by Collaborative Research, but Genzyme states that the mouse IL-2 was purified to homogeneity. $H_3^{32}PO_4$ was obtained from New England Nuclear (Boston, MA, U.S.A.). Fischer's medium (for leukaemic mouse cells), fetal bovine serum and antibiotics were purchased from GIBCO (Grand Island, NY, U.S.A.), and horse serum was purchased from Flow Laboratories (McLean, VA, U.S.A.). Sodium vanadate, NaF , $Na_4P_2O_7$ and PMA were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Cell culture

The Nb2 cells used in this study were derived from a clone (11C) produced in the laboratory of Dr. H. G.

Abbreviations used: IL-2, interleukin 2; PMA, phorbol 12-myristate 13-acetate; hGH, human growth hormone.

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Friesen. These cells were recloned by limiting dilution, and a new clone (BS/11C) showing high responsiveness to rat IL-2 was selected. The Nb2 cells were grown in Fischer's medium supplemented with 10% (v/v) fetal bovine serum, 10% (v/v) horse serum, 1 mM-2-mercaptoethanol, 100 units of penicillin/ml and 100 µg of streptomycin/ml and were maintained in a humidified atmosphere of CO₂/air (1:19) at 37 °C.

Measurement of cell proliferation

Cell proliferation was quantified by the bioassay of Tanaka *et al.* [25]. Approx. 24 h before addition of test substances, the Nb2 cells were growth-arrested by removing fetal bovine serum from the medium (i.e., resuspended in assay medium). At 8 h before addition of test substances, the cells were resuspended in fresh assay medium. In preparation for each assay, the cells were collected by centrifugation (500 g for 3 min), resuspended in fresh assay medium at a concentration of approx. 1×10^5 cells/ml, and 2 ml samples were pipetted into 35 mm-diam. tissue-culture dishes. For studies involving the ART-18 antibody, assays were performed in a total volume of 0.1 ml in 96-well culture plates. After a 3-day culture period, cell suspensions were diluted to 10 ml with Isoton (Curtin Matheson Scientific, Indianapolis, IN, U.S.A.), and cell number was determined with a Coulter counter (Coulter Electronics, Hialeah, FL, U.S.A.).

Anti-(IL-2 receptor) antibody

The monoclonal antibody specific for the rat IL-2 receptor (ART-18) was produced by Dr. T. Diamantstein [26]. The ART-18 antibody and a control monoclonal antibody were precipitated from ascitic fluid by adding (NH₄)₂SO₄ to 50% of saturation and centrifuging for 1 min in a Beckman Microfuge B. The precipitate was dissolved in Fischer's medium and washed twice with a Centricon 30 micro-concentrator (Amicon Corp., Danvers, MA, U.S.A.).

Radiolabelling of cells

Growth-arrested Nb2 cells (i.e. suspended in fresh assay medium 24 h and 8 h before use) were collected by centrifugation and resuspended in Fischer's medium in which 18 mM-Hepes was substituted for the normal phosphate buffer. The medium was supplemented with 0.1% bovine serum albumin, complete amino acids for RPMI 1640 medium, 2 mM-glutamine, 100 units of penicillin/ml and 100 µg of streptomycin/ml. The cells were centrifuged and resuspended in the same medium at a concentration of approx. 5×10^5 cells/ml. Then 1 ml portions of the suspension were dispensed into a 24-well culture plate, and the cells were immediately pulsed with [³²P]P_i at 0.05–0.1 mCi/well. After incubation of the cells at 37 °C in a humidified atmosphere of CO₂/air (1:19) for 0.5–1 h, test substances were added for the times indicated. Phosphorylation was terminated by suspending the Nb2 cells in 10 ml of a wash buffer containing 0.01 M-phosphate buffer (pH 7.4, 4 °C), 0.15 M-NaCl, 4 mM-EDTA, 10 mM-NaF, 0.1 mM-sodium vanadate and 10 mM-Na₄P₂O₇. The cells were collected by centrifugation, washed once more with the wash buffer, and heated to 100 °C for 3 min in a solution containing 2% SDS, 10% (v/v) glycerol and 2% (w/v) 2-mercaptoethanol. The solubilized cells were centrifuged to remove

insoluble material, and phosphorylated proteins were analysed by SDS/polyacrylamide-gel electrophoresis.

Electrophoresis and autoradiography

SDS/polyacrylamide-gel electrophoresis was performed by the method of Laemmli [27] by using a Bio-Rad vertical-slab-gel unit (Bio-Rad Laboratories, Richmond, CA, U.S.A.) with 1.5 mm-thick gels. The samples, containing equal amounts of radioactivity, were applied to a 14%- or 15%-polyacrylamide gel with a 4% stacking gel and were electrophoresed at 20 mA/gel. The gels were dried and autoradiographed on Kodak XAR-5 film (Picker International, St. Louis, MO, U.S.A.) by using enhancing screens. Pre-stained protein markers (Diversified Biotech, Newton Centre, MA, U.S.A.) were used for *M_r* determinations.

Densitometry

The extent of phosphorylation was quantified with a Multi-Media Densitometer (Gilford Instrument Laboratories, Oberlin, OH, U.S.A.). Small variations in the total amount of radioactivity applied were normalized by using as a reference a phosphoprotein that showed little difference in phosphorylation with or without the various substances. Phosphorylation in response to the various treatments was expressed as a percentage of that in untreated cells.

Statistical analysis

Statistical significance for potentiation of the actions of hGH and IL-2 by PMA was determined by Student's two-tailed *t* test for unpaired comparisons. The comparisons of several treatment means with a control were performed by Dunnett's test, and pairwise comparisons between treatment means were performed by the Newman-Keuls test. All data are expressed as means $\pm$ S.E.M.

RESULTS

Rat Nb2 lymphocytes proliferated in response to a partially purified preparation of rat IL-2 (Collaborative Research), as well as in response to hGH (Fig. 1). The rat IL-2 preparation used in this study stimulated proliferation in a dose-dependent manner and, at a concentration of 40 half-maximal units/ml, IL-2-stimulated proliferation was similar to that obtained with a maximally effective dose of hGH. Higher concentrations of the rat IL-2 preparation were not tested. The mitogenic action of hGH was potentiated by PMA ($P < 0.01$) when hGH was present at a concentration greater than 20 pg/ml (Fig. 1). PMA did not by itself stimulate proliferation. In the experiment shown in Fig. 1, PMA also potentiated the mitogenic action of rat IL-2, albeit to a lesser degree. The potentiation by PMA was significant ($P < 0.01$) at all but the 5-half-maximal-unit/ml dose of IL-2 ($P < 0.07$). These results have been confirmed in two subsequent experiments. Although the rat IL-2 was purified from cultures of rat splenocytes supplemented with fetal bovine serum, it is unlikely that a bovine prolactin contaminant was responsible for the mitogenic activity in the preparation, because IL-2-stimulated proliferation was not inhibited by an antiserum (anti-ovine prolactin, generously given by Dr. H. G. Friesen) capable of neutralizing the effects of 1 µg of bovine prolactin (results not shown). It is not possible at present to rule out effects of other contaminants (i.e. other lactogens or

Fig. 1. Proliferation of Nb2 cells in response to hGH (a) and to rat and mouse IL-2 (b)

Broken lines show the proliferative responses to these mitogens in the presence of 40 nM-PMA. In untreated groups, the Nb2-cell concentration was 1.9×10^4 cells/dish. Each point shows the mean of triplicate determinations and the error bars represent 1 S.E.M. No error bars are shown when the error is encompassed by the symbol. In (b): Δ , $\blacktriangle$, rat IL-2; $\square$, $\blacksquare$, mouse IL-2.

Fig. 2. Effect of a monoclonal antibody specific for the rat IL-2 receptor [26] on IL-2-stimulated (a) and hGH-stimulated (b) cell proliferation

The hGH (1 ng/ml), rat IL-2 (20 half-maximal units/ml) and antibody ($\square$ ART-18; $\blacksquare$, control) were added as indicated in a total assay volume of 0.1 ml. Each bar shows the mean of triplicate determinations, and the error bars represent ± 1 S.E.M.

growth factors); however, two lines of evidence suggest that IL-2, and not contaminants, was largely responsible for the observed stimulation of Nb2-cell proliferation. The primary evidence supportive of a mitogenic role for IL-2 is that a preparation of mouse IL-2 (Genzyme), produced by recombinant methodologies, also stimulated Nb2-cell proliferation. At a concentration of 400 units/ml,

the mouse IL-2 produced greater than a 2-fold increase in cell number (Fig. 1). As with rat IL-2, the response to mouse IL-2 was dose-dependent and was potentiated to a small degree by PMA. In the experiment shown in Fig. 1, the potentiation by PMA reached significance ($P < 0.02$) when mouse IL-2 was present at concentrations of 50 and 200 units/ml. The second line of evidence supporting a mitogenic role for IL-2 is that a monoclonal antibody specific for the rat IL-2 receptor (ART-18; [26]) decreased by more than 50% the IL-2-stimulated increase in cell number (Fig. 2). A non-specific antibody from control ascitic fluid did not affect proliferation in response to IL-2 or prolactin.

Uptake of ^{32}P into Nb2-cell protein was increased by approx. 40–80% by hGH, IL-2 and PMA. In order to identify changes in phosphorylation of specific proteins, equal amounts of radioactivity were applied to electrophoretic gels. Exposure of Nb2 cells to either hGH (Fig. 3, lane 3) or rat IL-2 (Fig. 3, lane 4) resulted in increased phosphorylation of an 18600-Da protein and in decreased phosphorylation of a 15600-Da protein relative to that in cells not exposed to mitogen (Fig. 3, lane 1). When autoradiographs from five separate experiments were analysed by densitometry, hGH and IL-2 appeared to be similarly effective in altering phosphorylation of the 15600-Da and 18600-Da proteins (Table 1), although the response to each mitogen in individual experiments was variable. The mouse IL-2 preparation, at a concentration of 400 units/ml, also increased phosphorylation of the 18600-Da protein and decreased phosphorylation of the 15600-Da protein (Fig. 4, lane 2). PMA, like hGH and IL-2, inhibited phosphorylation of the 15600-Da protein (Fig. 3, lane 2; Table 2). PMA did not stimulate phosphorylation of the 18600-Da protein, but it potentiated the actions of hGH ($P < 0.05$) on phosphorylation of the 18600-Da protein (Table 2). PMA also appeared to increase phosphorylation of the 18600-Da protein in response to IL-2, but the increase was not statistically significant. The effects of hGH and IL-2 on phosphorylation of the 15600-Da and 18600-Da proteins were not detected before 2 h (Fig. 5). Stimulation by hGH of phosphorylation of the 18600-Da protein reached a plateau at 3–4 h, whereas the inhibitory effects of hGH on phosphorylation of the 15600-Da protein began to

Fig. 3. Analysis by SDS/polyacrylamide (14%)-gel electrophoresis and autoradiography of protein phosphorylation in untreated Nb2 cells (lane 1) and in cells exposed to PMA (40 nM; lane 2), hGH (5 ng/ml; lane 3) and rat IL-2 (40 half-maximal units/ml; lane 4).

The cells were incubated with test substances for 4 h. The autoradiograph was over-exposed to show more clearly the effects of the various agents on phosphorylation of the 15600-Dalton protein. The numbers at the right are estimates of the M_r values ($\times 10^{-3}$) of the two phosphoproteins indicated by the arrows. The prestained M_r standards (Diversified Biotech) used in the electrophoresis were as follows: cytochrome *c* (12400), lactoglobulin (18400), carbonic anhydrase (29000), lactate dehydrogenase (36000), ovalbumin (43000), glutamate dehydrogenase (55000) and phosphorylase *b* (95000).

reach a plateau at 4 h (Fig. 5). Rat IL-2-stimulated phosphorylation of the 18600-Da protein appeared to increase between 3 and 4 h; however, the values at the two time points were not significantly different ($P < 0.1$). The time-course experiments were not extended beyond 4 h; thus it was not determined whether inhibition by IL-2 of phosphorylation of the 15600-Da protein was maximal at 4 h.

DISCUSSION

These are the first data to suggest that IL-2, as well as lactogens, directly stimulates proliferation of Nb2 cells. It is somewhat surprising that Nb2 cells chronically express receptors for IL-2, because expression of these receptors in T cells generally must be preceded by some type of 'activation' event [1]. However, chronic expression of the IL-2 receptor may reflect the 'immaturity'

Table 1. Effect of hGH and rat IL-2 on phosphorylation of 15600- and 18600-Da proteins

Phosphorylation is expressed as a percentage of that in untreated Nb2 cells (i.e. control = 100%); each value is the mean $\pm$ 1 S.E.M. for five experiments. * $P < 0.05$; ** $P < 0.01$.

Phosphoprotein	hGH (5 ng/ml)	Rat IL-2 (40 half-maximal units/ml)
15600 Da	53.2 $\pm$ 22.6*	54.3 $\pm$ 5.1**
18600 Da	222 $\pm$ 20.2**	238 $\pm$ 22.6**

Fig. 4. Analysis by SDS/polyacrylamide (15%)-gel electrophoresis and autoradiography of protein phosphorylation in untreated cells (lane 1) and in cells exposed to mouse IL-2 (400 units/ml; lane 2) and rat IL-2 (40 half-maximal units/ml; lane 3).

The cells were exposed to test substances for 4 h. The numbers at the left are estimates of the M_r values ($\times 10^{-3}$) of the two phosphoproteins indicated by the arrows; see Fig. 3 for M_r standards.

[9] of these cells. A substantial portion, if not all, of the mitogenic activity in the rat IL-2 preparation appears to be mediated through IL-2 receptors, inasmuch as the actions of IL-2 were inhibited by a monoclonal antibody specific for the rat IL-2 receptor [26]. Furthermore, mouse IL-2 prepared by recombinant methodologies, a preparation unlikely to contain mitogenic contaminants, also stimulated Nb2-cell proliferation, albeit to a lesser degree. The low activity of the mouse IL-2 was somewhat unexpected, because the activity of the rat IL-2 prep-

Table 2. Effect of PMA on hGH- and IL-2-induced phosphorylation

Phosphorylation is expressed as a percentage of that in untreated Nb2 cells (i.e. control = 100 %); each value is the mean of two experiments (numbers in parentheses represent ± 1 S.E.M.).

Phosphoprotein	hGH (5 ng/ml)	IL-2 (40 half- maximal units/ml)	PMA (40 nM)	GH + PMA	IL-2 + PMA
15 600 Da	78.0 (32.6)	56.5 (7.0)	48.5 (5.6)	52.0 (6.0)	40.5 (3.5)
18 600 Da	185 (7.0)	245 (11.0)	127 (32.3)	266 (17.0)	295 (23.1)

Fig. 5. Time course of phosphorylation of 15600-Da (○, △) and 18600-Da (●, ▲) proteins in response to hGH (5 ng/ml) and rat IL-2 (40 half-maximal units/ml)

Autoradiographs of SDS/polyacrylamide (15 %) gels were quantified by using a densitometer, and incorporation of radioactive phosphate in response to hGH and rat IL-2 was expressed as a percentage of that in untreated cells. Each point shows the mean of three separate experiments, and error bars represent ± 1 S.E.M.

aration in stimulating Nb2-cell growth was in accordance with the biological potency determined by the distributor. It is possible, however, that mouse IL-2 prepared by recombinant techniques differs from the native lymphokine or that the rat IL-2 receptor on Nb2 cells shows some species specificity. A preliminary report [28] has suggested that one role of IL-2 in Nb2 cells is to sensitize the cells to the mitogenic actions of Prolactin. A permissive action of this type probably cannot account for the potent mitogenic actions of the rat IL-2 in Nb2 cells, because the horse serum used in the Nb2-cell cultures contained undetectable amounts of lactogen. It cannot be totally discounted, however, that rat IL-2 sensitized Nb2 cells to a lactogen contaminating the

lymphokine preparation. For this to be the case, the putative lactogen could not be immunologically related to bovine prolactin, because antibodies capable of neutralizing this prolactin did not decrease the activity of the IL-2 preparation. It is also conceivable that a non-lactogenic contaminant in the rat lymphokine preparation, whose action affects or is affected by IL-2, could have augmented the potency of the rat IL-2 preparation, a possibility that is difficult to exclude until highly purified rat IL-2 is available. In any case, the results obtained with the mouse and the rat lymphokine preparations strongly support a mitogenic role for IL-2 in Nb2 cells.

To our knowledge, the results of the experiments on protein phosphorylation are the first to suggest that lactogens, IL-2 and PMA may regulate the function of the same proteins. It is unknown, at present, whether the observed changes in protein phosphorylation are involved in mediating the mitogenic actions of hGH and IL-2; however, the data are consistent with such a role. The phosphorylation events occurred within the interval over which hGH [13] and IL-2 [29,30] must be in contact with their receptors in order to trigger irreversible progression to DNA replication, and, at concentrations shown to induce near-maximal proliferative responses, hGH and IL-2 produced similar changes in protein phosphorylation. Moreover, phosphorylation of the 18600-Da protein in response to PMA also paralleled cell proliferation in response to this phorbol ester in that PMA appeared to augment the actions of hGH, but it had no effect in the absence of this mitogen. The one event shared by hGH, IL-2 and PMA was inhibition of phosphorylation of the 15600-Da protein; thus it is tempting to speculate that this protein may play a pivotal role in some aspect of T-cell regulation.

The phosphorylation events appear to be direct rather than permissive actions of IL-2 and hGH, because the phosphorylation experiments were performed in the absence of serum. Contaminants, though perhaps a problem in the rat IL-2 preparation, cannot account for the activity of the highly purified mouse IL-2 produced by recombinant techniques. Thus IL-2 appears to regulate phosphorylation in Nb2 cells, as it does in other T-lymphocytes [21,22]. A preliminary report [31] has suggested that lactogens also regulate phosphorylation of a 33000-Da protein in Nb2 cells, and it is likely that future studies will show that phosphorylation of additional proteins also is regulated by hGH and IL-2.

The actions of IL-2 and lactogens in Nb2 cells may be

mediated, in part, through activation of kinase C. The importance of kinase C in proliferation of Nb2 cells is underscored by the fact that an inhibitor of this enzyme decreases prolactin-induced thymidine uptake. Moreover, a potent activator of kinase C (i.e. PMA) potentiates the mitogenic actions of prolactin and IL-2 and mimics the actions of these mitogens on phosphorylation of the 15600-Da protein. These data are consistent with previous studies showing that IL-2 stimulates translocation of kinase C in lymphocytes [16], an action that may be secondary to activation of the phosphatidylinositol system [32]. It is unlikely, however, that activation of kinase C can account for all the effects of hGH and IL-2, inasmuch as PMA does not totally mimic the actions of these mitogens.

IL-2 and lactogens have been shown to regulate a number of intracellular events in T lymphocytes. One of the earliest T-cell responses to hGH [13] and IL-2 [14] is an increase in mRNA for the proto-oncogene *c-myc*; thus this proto-oncogene may serve as an important signal in T-cell proliferation. Additional actions of lactogens in Nb2 cells include stimulation of ornithine decarboxylase activity [10–12], regulation of prostaglandin synthesis [33], mobilization of Ca^{2+} [23] and increasing mRNA coding for a heat-shock-like protein [34]. At present, the interconnections between these intracellular events remain undefined, but further examination of these mechanisms in Nb2 cells should help to elucidate the interactions between lactogens and IL-2 in regulating immune function.

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