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Isolation of a Polypeptide That Has Lymphocyte-Differentiating Properties and Is Probably Represented Universally in Living Cells

(β-adrenergic receptors/cyclic AMP/radioimmunoassay/thymus leukemia antigen/complement receptors)

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ABSTRACT **A polypeptide of 8500 molecular weight is described that induces the differentiation of T (thymus-derived) cell and B (bone-marrow-derived) cell immunocytes *in vitro*, apparently via β-adrenergic receptors and adenylate cyclase activation. This polypeptide shows a high degree of evolutionary conservation, exhibiting close structural, functional, and immunological similarity when isolated from such diverse origins as cells of mammals and higher plants. This polypeptide was detected in animal cells, yeast, bacteria, and higher plants, and so may well be a universal constituent of living cells.**

The isolation from bovine thymus of the polypeptide hormones thymopoietin I and II has been described previously (1 2). [We are now using the term thymopoietin (3) because valid objections (4) were raised to our previous term "thymin," which is easily confused with the pyrimidine base thymine.] Pure thymopoietin I and II specifically induce prothymocytes to differentiate into thymocytes (5–7), but these polypeptides were recognized originally not by this evidently physiological faculty of T (thymus-derived) cell differentiation but by a presumably incidental property discovered in the course of studies related to the human disease myasthenia gravis, in which a characteristic neuromuscular disturbance is consistently associated with thymic pathology. When reliable assays of T-cell differentiation *in vitro* became available, based on the manifestation of T-cell antigens on precursor cells (5, 8) it was realized that, although certain pure thymic products (thymopoietins) could induce this step in T-cell differentiation specifically, extracts of tissues other than thymus were also active (10).

We describe here the isolation and purification from bovine thymus of a polypeptide subsequently detected in many other tissues, which like thymopoietin induces the differentiation of pro-thymocytes, but which unlike thymopoietin induces differentiation of B (bone-marrow-derived) cells, and is thus distinguished in its mode of action from the physiological inducer thymopoietin. This new polypeptide has been provisionally named ubiquitous immunopoietic polypeptide (UBIP) because it is widespread and perhaps even universally represented in living cells and because one of its most useful properties for purposes of further study is its capacity to induce differentiation of T and B cells.

Abbreviations: UBIP, ubiquitous immunopoietic polypeptide; TL antigen, thymus leukemia antigen; Ig, immunoglobin; CR, complement receptor; CM-Sephadex, carboxymethyl-Sephadex; T, thymus-derived; B, bone-marrow-derived.

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Isolation of UBIP

Early stages in the isolation of the thymopoietin entailed the extraction from bovine thymus of polypeptides with molecular weights ranging from 4,000 to 12,000. Disc electrophoresis of this fraction on polyacrylamide gels revealed a major polypeptide component, and isolation methods were developed for this polypeptide with polyacrylamide disc electrophoresis being used to monitor purity. The polypeptide, which is UBIP, was isolated from bovine or human thymus, although we now know it to be present in other tissues (see below).

The isolation of UBIP from bovine thymus is summarized in Fig. 1. Bovine thymus was obtained fresh on wet ice, dissected clean and stored at −20°. Batches were homogenized 25% wet weight/volume in 0.1 M ammonium bicarbonate with a Waring blender. The extract was heated to 70° for 30 min and then centrifuged at 500 × *g* for 30 min. The supernatant was filtered through gauze and cotton and 0.1% thimerosal was added as a bacteriostatic agent, since the subsequent steps were carried out at room temperature. The extract was processed through a Diaflo XM100A membrane (Amicon) in a TCF10 apparatus. The dialysate was then concentrated over a Diaflo UM2 membrane at 55 lb./in.^{2}, using a 402 stirred cell and a reservoir; 3 liters were concentrated to 15 ml. This retentate was fractionated on a 2.5 × 100-cm column of medium Sephadex G-50 (Pharmacia) in 0.1 M ammonium bicarbonate. The fractions shown in Fig. 1 were lyophilized and rerun on the same column. The lyophilized fractions from this column were then fractionated by adsorption chromatography on hydroxyapatite (Bio-Rad). A 500-mg load was dissolved in 5 mM sodium phosphate buffer, pH 6.8, and applied on a 2.5 × 30-cm column of hydroxyapatite in the same buffer. Thymopoietin eluted behind the void volume with 5 mM phosphate buffer; subsequently the concentration of the eluting buffer was raised to 50 mM. The fractions shown in Fig. 1 were lyophilized and desalted on Sephadex G-25 in 0.1 M ammonium bicarbonate. The materials eluting at the void volume of the G-25 column were lyophilized and taken up in 0.2 M ammonium acetate which had been brought to pH 4.5 with acetic acid, and the sample was applied to a 0.6 × 30-cm column on CM-Sephadex in the same solvent. This was developed with a linear gradient to 0.55 M ammonium acetate, pH 4.7. The fractions shown in Fig. 1 were lyophilized and desalted on Sephadex G-25 in 0.1 M ammonium bicar-

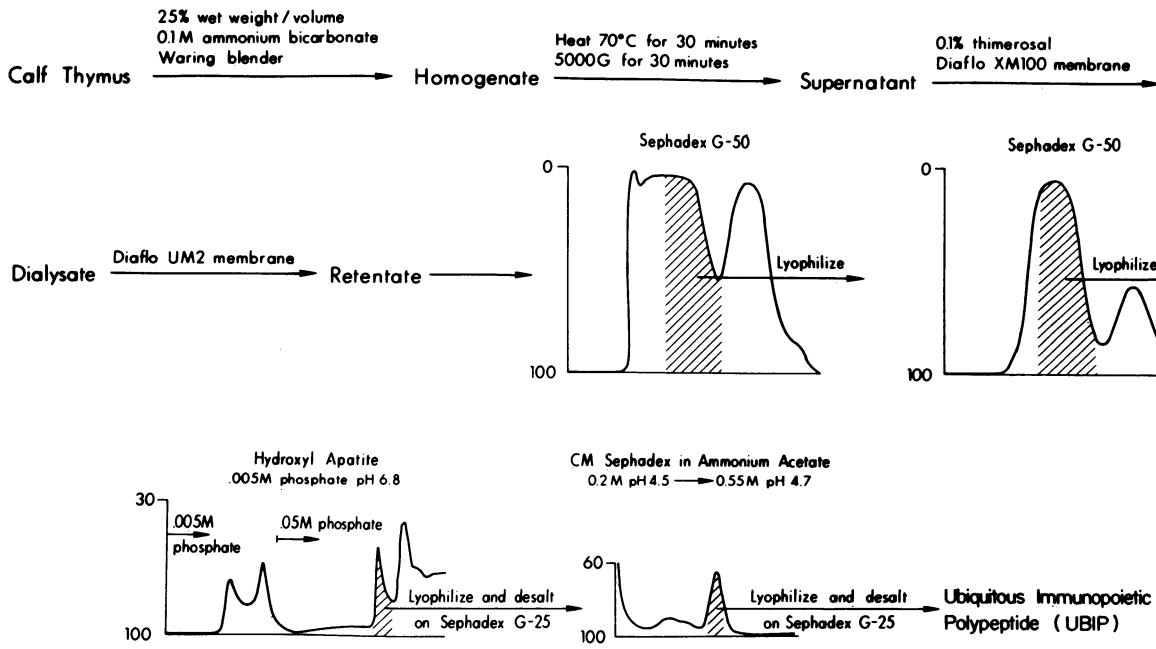

FIG. 1. Flow chart showing steps in the isolation of UBIP from bovine thymus. The chromatographic elution profiles are of percent transmittance at 254 nm and the shaded areas represent the fractions collected.

bonate, and the lyophilized void volume material constituted purified UBIP.

Purity of the UBIP preparations was assessed by polyacrylamide disc electrophoresis at pH 8.9 and pH 4.3 as described previously (1). With a load of 0.2 mg per gel there was a single band at both pH 8.9 and pH 4.3 (Fig. 2). The relative mobility (R_F) with respect to bromphenol blue was 0.26 at pH 8.9 and the R_F with respect to methyl green was 0.76 at pH 4.3. Furthermore, amino-acid sequence studies of UBIP show it to consist of a single polypeptide chain containing 74 amino-acid residues with methionine at the N-terminus and arginine at the C-terminus (D. H. Schlesinger, G. Goldstein, and H. D. Niall, manuscript in preparation).

The molecular weight of UBIP was estimated to be 8500 on the basis of molecular exclusion chromatography, amino-acid composition, and amino-acid sequence. It was not possible to calculate recoveries or enrichment at each step of the isolation because purification was based solely on polyacrylamide disc electrophoresis criteria. On the average, 1 kg of wet thymus yielded 30 mg of UBIP.

Induction of T-cell and B-cell differentiation by UBIP

UBIP causes no neuromuscular lesion detectable in the electromyographic assay originally used to monitor the isolation of thymopoietin. UBIP did, however, induce the differentiation of immunocyte precursors *in vitro*, although the following data show that activity in this respect is clearly distinguishable from that of thymopoietin.

The differentiation of pro-thymocytes to thymocytes was assayed by the acquisition of the thymic differentiation antigen TL (thymus leukemia) (5, 8) using the Komuro–Boyse assay in which the induced (now TL^+) cells are lysed on exposure to TL antiserum and complement in the cytotoxicity test (8). The differentiation of B cells was measured under similar conditions *in vitro*. In the experiments reported here the acquisition of receptors for complement (CR) was used as an index of B-cell differentiation (7, 11); the precursor B cells were derived from fractionated mouse spleen cells as in the case of pro-thymocytes.

We recognize that these T-cell and B-cell differentiation assays are not strictly comparable because TL conversion and acquisition of C3 receptors are not strictly equivalent events in the sequence of T-cell and B-cell maturation. Nevertheless the CR^- to CR^+ conversion is a clearly defined step in B-cell differentiation as is the TL^- to TL^+ conversion of the pro-thymocyte. Other markers for sequential events in the B cell differentiation (surface Ig^- to Ig^+, or acquisition of B-cell surface antigens) can equally be employed, and we have done so; use of the CR criterion in this study was a matter of convenience.

The results are summarized in Table 1. UBIP induced *in vitro* the differentiation of pro-thymocytes (14% cells induced to express TL at 1 ng/ml) and pro-B cells (10% cells induced to express CR at 1 ng/ml). By contrast, thymopoietin induced pro-thymocyte differentiation (13% cells induced to express TL at 1 ng/ml) but had no effect on pro-B cells (no significant induction of CR^+ cells at 1, 10, and 100 ng/ml).

A further contrast between the inductive potentials of thymopoietin and UBIP is revealed by propranolol, which blocks β-adrenergic receptors. We know that the inductive potential of crude tissue extracts of thymus or other organs on both T cell and B cell differentiation can be blocked by propranolol (7). Propranolol, 10 μM, also blocked both T-cell and B-cell differentiation by UBIP, whereas the induction of T-cell differentiation by thymopoietin was not affected (Table 1), signifying that the initiating step by the specific (thymopoietin) and nonspecific (UBIP) inducers is dissimilar.

Representation of UBIP in cells of diverse origins

UBIP isolated from thymic extracts was studied in the first instance because it is the major polypeptide component in the 4,000 to 12,000 molecular weight range and, therefore, it seemed quite likely to be a thymic hormone in some form. We see that this is not so. Unlike thymopoietin, which is detectable only in thymus extracts and not extracts of other tissues (2, 12), UBIP has been detectable by disc electrophoresis with polyacrylamide gels in extracts from all guinea pig tissues tested (Fig. 3).

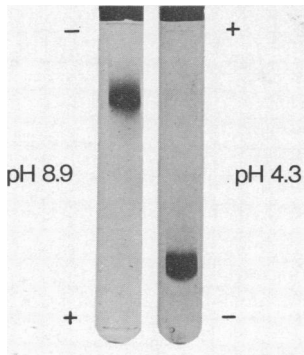

FIG. 2. Polyacrylamide gels from disc electrophoresis of 0.2-mg samples of UBIP. The gels were fixed with trichloroacetic acid and stained with Coomassie blue. One major band is present in each gel.

A radioimmunoassay for UBIP was devised to provide a ready method for its detection in tissue extracts. New Zealand rabbits were injected intradermally at multiple sites biweekly with Freund's complete adjuvant containing 0.5 mg of bovine UBIP coupled to an equal amount of horse gamma globulin by glutaraldehyde (13). Adequate titers developed after four to eight injections. UBIP was labeled with ^{125}I by the chloramine-T procedure (14) and labeled polypeptide was separated from free iodine by gel filtration on Sephadex G-25; the incorporation of ^{125}I was usually about 80–90% and separation of the iodinated polypeptide from free iodine was complete.

In the absence of unlabeled UBIP, this UBIP antiserum at a dilution of 1:10 bound about 90% of labeled UBIP and about 50% at a final antiserum dilution of 1:2000. The nonspecific binding in the presence of excess unlabeled UBIP, or after incubation of tracer with a nonimmune serum, was 10%. Fig. 4 shows the binding-inhibition curve obtained with unlabeled bovine and human UBIP, each of which produced substantial displacement of labeled UBIP in the range of 1–100 ng. The binding-inhibition curves of human and bovine UBIP were identical, indicating complete immunological cross reactivity between the human and bovine polypeptides.

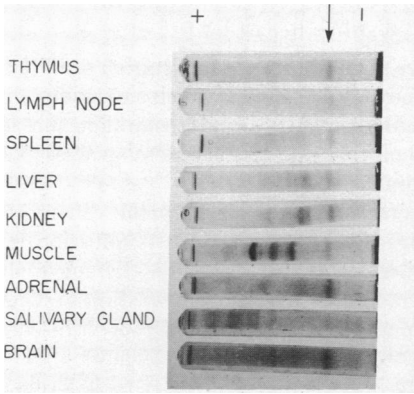

FIG. 3. Polyacrylamide gels from disc electrophoresis at pH 8.9 of 0.5-mg samples of extracts of various guinea pig tissues. Each tissue was processed through the Sephadex G-50 step of the isolation (see *text* and Fig. 1) so that a fraction containing polypeptides of 4,000 to 12,000 molecular weight was prepared. The arrow indicates the mobility of UBIP and a stained band is present in each gel at this position. In gels run at pH 4.3, a band with the mobility of UBIP was similarly found for each tissue, so that each tissue contained a major polypeptide component with the same mobility as UBIP at both pH 4.3 and pH 8.9

TABLE 1. *Induction of immunocyte differentiation in vitro*

Polypeptide	Concentration (ng/ml)	Propranolol, 10 μM	Induction of cytotypic marker, induction index* TL antigen	Complement receptor
Thymopoietin	100	−	20 ± 4	<2
	10	−	17 ± 3	<2
	1	−	13 ± 3	<2
	10	+	16 ± 3	<2
UBIP	100	−	18 ± 3	16 ± 3
	10	−	18 ± 3	16 ± 3
	1	−	14 ± 3	10 ± 2
	10	+	<2	<2

Differentiation of immunocyte precursors *in vitro* induced by thymopoietin I or UBIP, according to the criteria of TL-antigen expression (T cells) and of expression of complement receptors (B cells). Spleen cells from A or congenic A/TL$^-$ mice were fractionated on bovine serum albumin gradients as described previously (8) and induction of cytotypic markers was studied in the B layer (23–26% interface), which was found to contain both the pro-thymocytes and immature B cells. TL induction was measured by the direct cytotoxic test with anti-TL antibody and complement as described previously (8). Induction of complement receptors was measured by the formation of rosettes with EAC sheep erythrocytes coated with subagglutinating quantities of rabbit antibody to sheep erythrocytes plus nonlytic complement (11). Before induction the complement receptor lymphocytes (CRL) in spleen were first depleted by forming rosettes as in the standard assay (11) and removing them by differential centrifugation; this was achieved by the 2-step bovine serum albumin gradient described by Bianco *et al.* (11) (free lymphocytes being collected at the 10–35% interface) or by low-speed centrifugation (free lymphocytes remaining in the supernatant). The CRL-depleted population was further enriched for B precursor cells by fractionation on a discontinuous 5-step serum albumin gradient (8) which gives an optimal yield at the 23–26% interface. This cell fraction was incubated with or without polypeptide for 2 hr and afterwards scored for CR$^+$ cells according to Bianco *et al.* (11).

* Mean ± SEM. Data represent summary of four experiments. Calculated from $[(a - b)/a] \times 100$ where a = % negative cells after incubation without polypeptide, and b = % negative cells after incubation with polypeptide.

Fig. 5 shows that a single peak of UBIP could be registered by radioimmunoassay in calf thymus homogenate fractioned by molecular sieve chromatography on Sephadex G-50. Thus, the numerous other substances in this extract did not compete with ^{125}I-labeled UBIP for the anti-UBIP antibody. Further specificity controls are shown in Table 2. Most proteins or polypeptides tested showed no detectable UBIP (<0.001%) or trivial amount (0.002–0.01%). Only ribonuclease B appeared to contain a significant amount of UBIP (0.1–1%). Contamination with UBIP rather than cross reactivity is the more likely explanation because two batches of ribonuclease B gave a 10-fold difference. Also, although in the case of the ribonuclease B sample with 1% UBIP the ribonuclease B band ran with the same mobility as UBIP in polyacrylamide gel electrophoresis at pH 4.3, the ribonuclease ran distinctly slower at pH 8.9, and a faster band with the mobility of UBIP was clearly stained.

This highly specific radioimmunoassay for UBIP was then

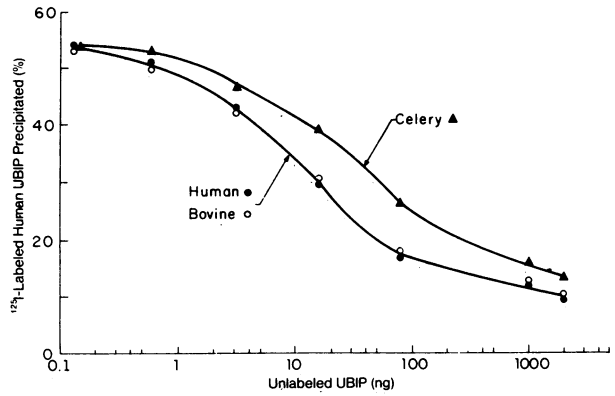

FIG. 4. Standard curves of binding inhibition for UBIP radioimmunoassay using ^{125}I-labeled human UBIP and comparing the curves obtained with unlabeled UBIP from man, cattle, and celery. The radioimmunoassay was carried out as follows: Incubations were carried out in 12 × 75-mm polystyrene tubes, employing a diluent solution of bovine gamma globulin (5 mg/ml) in phosphate-buffered saline. Each determination was made in duplicate or triplicate. To each tube was added 0.5 ml of 1/1000 antiserum to UBIP, 0.2 ml of diluent, UBIP standards, or test extract, and 0.3 ml of (50,000 cpm) ^{125}I-labeled human UBIP.

After incubation of the assay tubes at room temperature for 2 hr, antibody-bound and free UBIP were separated by precipitation of the bound tracer with 10% polyethylene glycol (15); 0.5 ml of cold 30% polyethylene glycol was added to each tube, and the precipitate was sedimented by centrifugation at 900 × *g* for 30 min. The supernatant solutions were aspirated, and the precipitated radioactivity in each tube was determined with an automatic gamma spectrometer (Nuclear Chicago 1185).

used to determine further the distribution of UBIP in other cells. The results are summarized in Table 3. The widespread occurrence of UBIP in mammalian tissues indicated by disc electrophoresis on polyacrylamide gels was confirmed. UBIP was present in the tissues of (*nu/nu*) mice, which have no thymus, mammalian tumor cell lines, fibroblasts, various cells maintained in culture, and avian embryos and fibroblasts in culture. The phylogenetic pedigree of UBIP according to the radioimmunoassay suggests that UBIP is perhaps ubiquitous in living cells, because it is found not only in fish and a nonvertebrate but also in higher plants, yeasts, and bacteria (Table 3). A small quantity of UBIP was isolated from celery, as representing a phylogenetic source extremely distant from

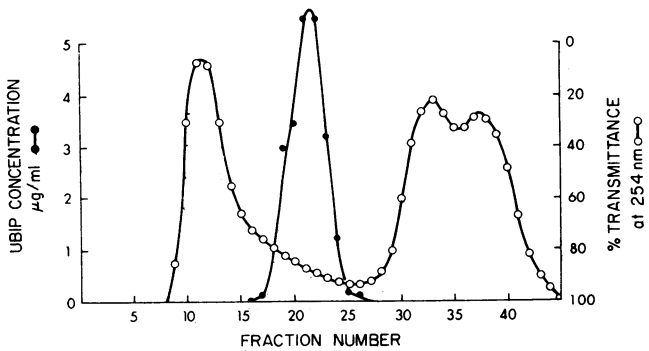

FIG. 5. Radioimmunoassay detection of UBIP in calf thymus homogenate fractionated by molecular sieve chromatography on Sephadex G-50. A sharp peak of UBIP is found in the fractionation range of the column and there is no displacement in the radioimmunoassay by the materials producing the high absorbance peaks at the void volume, V_0, or included volume, V_i.

TABLE 2. *Percent of UBIP by weight detectable by radioimmunoassay in various proteins or polypeptides*

UBIP not detectable (<0.001%): Maltase (Sigma), lactoperoxidase (Calbiochem), β-glucosidase (Worthington), chymotrypsinogen A (Mann), insulin (Sigma), cytochrome *c* (Mann), bacitracin (Mann), glucagon (Sigma), myoglobin (Mann), ovalbumin (Mann), thymopoietin I (own lab).

UBIP detectable: Thymopoietin II (own lab), 0.002%; α-chymotrypsin (Worthington), 0.01%; apoferritin (Mann), 0.01%; ribonuclease B (Mann), 0.1%; ribonuclease B (Worthington), 1.0%.

The findings with ribonuclease B probably represent contamination of the preparation with UBIP (see *text*) and the absence or trivial levels of UBIP found with other proteins or polypeptides emphasizes the specificity of the assay.

mammals. The isolation methods were as for bovine UBIP except that the celery was initially homogenized in a domestic "juicer" and centrifuged, the heating step was omitted, and the position of celery UBIP on CM-Sephadex chromatography was determined by radioimmunoassay because the amounts recovered were too small to produce a detectable peak of ultraviolet absorbance.

Amino-acid sequence at the N-terminus was determined by sequential Edman degradation on an automated sequencer as described (16). There is a remarkable conservation of amino-acid sequence in UBIP, six of the first eight residues being identical in bovine and celery UBIP (Fig. 6). This work was done on a small amount of material and it must be confirmed and extended.

Additional evidence for structural conservation comes from the radioimmunoassay, in which the binding-inhibition curve of celery UBIP is very similar to that of human and bovine UBIP (Fig. 4). Functional tests provide further evidence of conservation. Celery UBIP, like bovine UBIP, induced *in vitro* the differentiation of mouse pro-thymocytes (9% induction of TL^+ cells) and B cells (16% induction of CR^+ cells), and both effects were inhibited by 10 μM propranolol (<2% induction of TL^+ and CR^+ cells).

Biological significance of UBIP

As we have pointed out, the recognition of UBIP was incidental to a study on the thymus which had a quite different objective. But the properties and remarkable phylogenetic conservation of this polypeptide raise general points that are worthy of note.

Thymopoietin induces *in vitro* the differentiation of prothymocytes to thymocytes. This induction, evidently mediated via cAMP (7), is specific for thymocyte differentiation, since thymopoietin has no demonstrable effect on the differentiation of B cells (6, 7), erythropoietin-sensitive cells (6), or pluripotent hemopoietic stem cells (6). UBIP, on the other hand, induces the differentiation of both T cells and B cells *in vitro* and this is prevented by the β-adrenergic blocking agent propranolol. This clearly suggests that the two differentiation events are similarly governed by cyclic AMP-dependent regulatory mechanisms, which can be activated through β-adrenergic receptors. Furthermore, these findings suggest discrete immediate precursors for T cells and CR^+ B cells, the former recognizing thymopoietin and the latter not, and this is fully in accord with previous conclusions that both differen-

TABLE 3. *UBIP levels (μg/g) in various tissues and cells as measured by radioimmunoassay*

Guinea pig: lymph node 125, spleen 100, liver 100, kidney 65, muscle 35, salivary gland 25, thymus 5. *Mouse, outbred:* muscle 3, kidney 15, liver 4, ASCI (T cell leukemia) 4, RADI (T cell leukemia) 50, 3T3 fibroblasts (tissue culture) 2, SV101 fibroblasts (tissue culture, transformed) 32. *Mouse (nu/nu), athymic:* muscle 30, kidney 15, liver 5. *Chicken:* 10 day embryo 22, 13 day embryo 45, fibroblasts (tissue culture) 32. *Hamster,* BHK kidney (tissue culture) 10. *Fish (perch)* muscle 18. *Squid* muscle 100. *Celery* stalk 0.11. *Eggplant* fruit 0.05. *Carrot* root 0.14. *Pear* fruit 0.05. *Apple* fruit <0.05.

Yeast, brewers bottom 10. *Candida utilis (torula)* 10. *Escherichia coli* (ATCC 8739) 1.2, strain W (ATCC 9637) 1.8, strain B (ATCC 11303) 0.3, strain K12 0.3. *Bacillus subtilis* (ATCC 6633) 0.4. *Clostridium histolyticum* (ATCC 8034) <0.05. *Clostridium kluyveri* (grown on synthetic media) 0.2.

Tissues or cell culture lines were homogenized in 10 parts of phosphate-buffered saline containing 5 mg/ml of bovine gamma globulin. The homogenate was centrifuged and the supernatant tested in the radioimmunoassay undiluted and in 10-fold dilutions, the binding-inhibition curve being compared with that of UBIP standards. The amount of UBIP is expressed as μg/g of wet weight. The yeasts and bacteria, which were obtained lyophilized, were first suspended and washed in the buffer to remove any remaining nutrient medium. After washing, the pellet was resuspended and homogenized in the presence of glass beads to disrupt cells. After centrifugation the supernatant was tested in the radioimmunoassay as above. The amount of UBIP for these is expressed as μg/g of dry weight.

tiative steps concern the triggering of pre-programmed or "committed" precursors.

The finding that UBIP, a potent inducer of immunocyte differentiation, is widespread and perhaps ubiquitous in cells of all kinds emphasizes the crucial role of specificity controls in the studies of physiological differentiation or induction of any kind, not least in experimental embryology, where the long search for specific inducers has been hampered by positive results with incongruous tissues or other materials (17–20).

Although UBIP is a potent inducer of immunocyte differentiation *in vitro*, it is unlikely to have such a function *in vivo*. Athymic (*nu/nu*) mice have plentiful pro-thymocytes and they characteristically lack T cells, but their tissues contain normal quantities of UBIP.

What, then, is the physiologic function of UBIP, since UBIP from such phylogenetically disparate sources as mammals and plants shows such uniformity by structural, functional, and immunological criteria? That UBIP should have been so rigorously conserved throughout this immense evolutionary time-span suggests a function vital to the living organism. As UBIP is present in bacteria, its function clearly antedates even such early evolutionary steps as karyon formation, let alone the cellular organization of metazoa and intercellular regulation by nerve networks and endocrine-receptor signals. UBIP activates adenylate cyclase in a wide variety of

	1	2	3	4	5	6	7	8
Bovine UBIP	NH_2-Met-	Gln-	Ile-	Phe-	Val-	Lys-	Thr-	Leu-
Celery UBIP	NH_2-Asp-	Gln-	Ile-		Val-	Lys-	Thr-	Leu-

FIG. 6. The amino-acid sequence of bovine and celery UBIP at the N-terminus. Position 4 of celery UBIP was not identified and there is a substitution at position 1; the remaining residues are identical.

tissues (M. W. Bitensky and G. Goldstein, in preparation), and it may function physiologically as an essential element of this basic mechanism, since adenylate cyclase is also very widely represented in animals, plants, and unicellular organisms (21).

However that might be, we can infer that the function of UBIP is an integral feature of living cells. The nature of that basic function is unknown, and this provides a question that may have intrinsic importance in its own right. Likewise, the way in which UBIP triggers pre-programmed immunocytes and doubtless other committed cells remains to be elucidated in detail. This itself may be of considerable value in further studies of differentiative-inductive processes generally (20).

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