Other fusion events beside those that occur between two populations of early endosomes have been reconstituted. Plasma membrane–derived vesicles, generated by homogenization of cells whose external Fc receptors were saturated with aggregated anti-DNP IgG, can also fuse with early endosomes in a cell-free system (*12*). This process mediates the transport of cell-surface molecules to an endocytic compartment. Endosomes contain acid proteases that can digest internalized proteins (*13*), and therefore, the fusion of vesicles generated shortly after internalization (which do not contain proteases) with protease-containing compartments (that is, endosomes and probably also Golgi-derived vesicles) should trigger the proteolytic processing of internalized ligands. This fusion-dependent proteolysis can be reconstituted and monitored by the onset of degradation of an internalized radiolabeled protein (*14*). Fusion between plasma membrane–derived vesicles and endosomes and fusion-dependent proteolysis were both inhibited by GTP-γ-S (half-maximal inhibition at 1 to 3 μ*M* GTP-γ-S), and the effect was completely reversed by 1 m*M* GTP (Fig. 1, B and C). Moreover, the inhibitory effect was only observed at high concentrations of cytosol (Fig. 3, B and C).

Cell-free reconstitutions of fusion processes of both the exocytotic and endocytic pathways have several common requirements. Fusion requires cytosolic proteins and energy in the form of ATP (*10, 11, 15*). In vitro transport requires an *N*-ethylmaleimide–sensitive factor (*11, 15*). This factor has been purified to homogeneity (*16*) and shown to be required in both the secretory and the endocytic pathways (*17*). We show here that a putative GTP-binding protein may also participate in the regulation of fusion events after receptor-mediated endocytosis. Therefore, a common mechanism may support fusion in the endocytic and exocytotic pathways. However, some specific components must exist to avoid transport to inappropriate compartments and to allow individual modulation of both pathways. Distinctive markers on the limiting membrane of the compartments and specific regulatory components of the fusion process may account for the independence of the two pathways.

The exact role of GTP-binding proteins in intracellular transport is unknown. Most GTP-binding proteins are activated on binding GTP and become deactivated on hydrolysis of the nucleotide. This property may allow GTP-binding proteins to serve as regulatory components in vesicular transport. It may also provide a mechanism for coupling the energy of GTP hydrolysis to the transport process (*9*).

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Antigen-Specific Helper Function of Cell-Free T Cell Products Bearing TCR $V_\beta 8$ Determinants

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Although the T cell receptor (TCR) αβ heterodimer and its encoding genes have been characterized, a cell-free form of this receptor, which is needed for the study of functional or ligand-binding properties of the receptor, has not previously been isolated. When the cell-free supernatant products of activated cloned T helper (T_H) cells were found to mediate helper activity with antigen specificity identical to that of intact T cells, experiments were carried out to determine whether this functional activity was mediated by a cell-free form of TCR-related material. A disulfide-linked dimer indistinguishable from the T cell surface αβ heterodimer was precipitated from cell-free supernatants of cloned T_H cells with F23.1, a monoclonal antibody specific for a TCR $V_\beta 8$ determinant. Moreover, when cell-free T_H products were bound to and eluted from immobilized F23.1, these affinity-purified materials had antigen-specific and major histocompatibility complex–restricted helper activity that synergized with recombinant lymphokines in the generation of B cell antibody responses. These findings suggest that the factor isolated from T cell supernatants is a cell-free form of the TCRαβ dimer.

THE TCRαβ HETERODIMER HAS been identified, and both its glycoprotein structure (*1*) and its encoding genes (*2*) have been characterized. Recent DNA-mediated gene transfer experiments have directly demonstrated that this αβ dimer mediates the antigen-specific and major histocompatibility complex (MHC)–restricted recognition function of many helper and cytotoxic T cells (*3, 4*). To date, however, it has not been possible to assess the specificity or functional properties of the TCR in soluble or cell-free form. A system in which cell-free TCR material can be isolated and studied would permit analysis of the TCR-ligand interactions and could be used to test whether TCR structures can act as signaling molecules in the interaction of T cells with other cells. The cell-free products of T_H cell clones provide antigen-specific and MHC-restricted helper activity in the generation of hapten-specific immunoglobulin G (IgG) antibody responses (*5*). Because this specificity closely reproduces the recognition specificity of the intact T_H cells, presumably mediated by the αβ TCR, we studied the cell-free supernatants of activated T_H cells for the presence of TCR-like products.

Cloned antigen-specific and MHC-restricted type 2 T_H cells (*6*), which secrete interleukin-4 (IL-4) but not IL-2 (*7*), were activated in the presence of specific antigen and appropriate antigen-presenting cells

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(APC). The activated T_H cells generated supernatants that supported trinitrophenyl (TNP)-specific IgG antibody responses by TNP-primed B cells in the absence of intact T cells. Maximal antibody responses were generally observed at supernatant dilutions of 10^{-2} or 10^{-3}. The helper activity provided by T_H clone supernatants was found after extensive titrations to be comparable to that provided by an equivalent number of intact T_H cells (*5*). The help mediated by these supernatants was both antigen-specific and MHC-restricted. Clone 2-19-2, which is specific for fowl gamma globulin (FGG) + $A_\alpha^b A_\beta^b$, generated a supernatant that supported responses of TNP-primed B10 ($I\text{-}A^b$) B cells to TNP-FGG but not to TNP-keyhole limpet hemocyanin (KLH) (Fig. 1A). Clone A-8, which is specific for KLH + $A_\alpha^k A_\beta^k$, generated a supernatant that supported responses of TNP-primed B10.A ($I\text{-}A^k$) B cells to TNP-KLH but not to TNP–bovine serum albumin (BSA) + KLH (Fig. 1B). MHC restriction in the activity of these helper cell products was demonstrated by the ability of clone 8-5 (KLH + $A_\alpha^b A_\beta^b$) supernatant to help responses of B10 but not congenic B10.A strain B cells, and the reciprocal ability of clone A-12 (KLH + $A_\alpha^k A_\beta^k$) supernatant to help B10.A B cells but not B10 B cells (Fig. 1C). To determine whether antigen- and MHC-specific helper factor was a product of T cells and not a reflection of carryover of antigen or APC, we stimulated selected T_H clones by culturing them for 24 hours with monoclonal antibody (MAb) to CD3 (anti-CD3) (*8*) immobilized on a polystyrene surface, in the absence of antigen or APC. Supernatants generated in this fashion provided antigen- and MHC-specific helper activity comparable to that observed for antigen-induced supernatant (*5*).

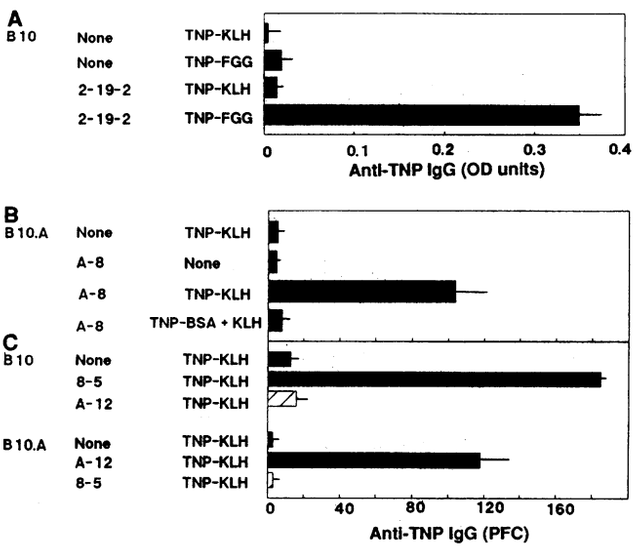

Fig. 1. Antigen specificity and MHC restriction of helper factor–mediated B cell activation. T cell–depleted spleen cells (1.5×10^6) from TNP-KLH–primed B10 or B10.A mice were cultured with 1:100 final dilution of supernatants from antigen-activated (**A**) FGG + $A_\alpha^b A_\beta^b$–specific clone 2-19-2, (**B**) KLH + $A_\alpha^k A_\beta^k$–specific clone A-8, and (**C**) KLH + $A_\alpha^b A_\beta^b$–specific clone 8.5 or KLH + A_α^k-A_β^k–specific clone A-12 in the presence of rIL-2 (50 U/ml) and rIL-4 (100 U/ml), with no antigen or with TNP-KLH, TNP-FGG, or TNP-BSA + KLH (0.1 μg/ml). Activation of clones by 24-hour culture with antigen-pulsed APC was performed as described (*5*). Production of IgG anti-TNP was measured by enzyme-linked immunosorbent assay (ELISA) (A) or plaque-forming cell (PFC) assay (B and C) as described (*5*) after culture of clone supernatant, T cell–depleted spleen B cells, lymphokines, and antigen. All ELISAs were performed by titrating culture supernatants, and the optical density (OD) values reported were obtained on a linear portion of the titration curves where optical density was proportional to supernatant concentration. All data are presented as mean ± SEM of triplicate cultures.

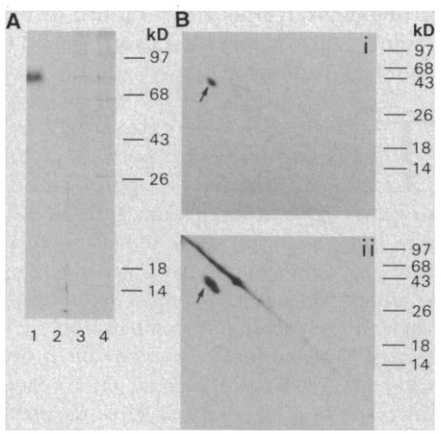

Fig. 2. Immunoprecipitation of supernatants and cell lysates from T_H clones. (**A**) Polyacrylamide gel analysis of immunoprecipitates of T_H clones 10-5-17 ($V_\beta 8^+$) (lanes 1 and 2) and 8-5 ($V_\beta 8^-$) (lanes 3 and 4) with TCR $V_\beta 8$–specific MAb F23.1 (lanes 1 and 3) or an irrelevant IgG2aK MAb 22 (*19*) (lanes 2 and 4). (**B**) Nonreducing-reducing two-dimensional gel electrophoresis of MAb F23.1 immunoprecipitates from T_H cell supernatants (i) and cell lysates (ii) of clone 10-5-17. Cells (2×10^7) were cultured as described (*5*) in the presence of immobilized antibody 2C11 directed against CD3ε. Cells were metabolically labeled by incubation at 37°C with 2 mCi of [^{35}S]methionine (1100 Ci/mmol) (Dupont–New England Nuclear) in 15 ml of methionine-free RPMI 1640 containing 2% fetal calf serum. After 9 hours, cell supernatant was removed and centrifuged at 250*g* for 10 min. The cell monolayer was washed and solubilized in 0.6 ml of Triton X-100 lysis buffer (*20*). Supernatants (3 ml) and lysates (0.2 ml) were incubated with 5 μg of affinity-purified monoclonal antibody (F23.1 or MAb22) overnight at 4°C. Immune complexes were then collected by incubation with protein A–Sepharose (Pharmacia), washed, and solubilized (*20*) under nonreducing conditions. In (A) SDS-polyacrylamide gel electrophoresis was performed as described (*21*) with the use of 12.5% gels under nonreducing conditions. In (B), two-dimensional gel analysis was performed by electrophoresis in 12.5% SDS-polyacrylamide tube gels under nonreducing conditions and was followed by equilibration with 0.5% dithiothreitol in 0.1% SDS; the reduced proteins were separated by electrophoresis in 15% SDS-polyacrylamide gels. The gels were fixed, incubated with EN^{3}HANCE (Dupont–New England Nuclear), and subjected to fluorography at −70°C. The arrows in (B) indicate the position of the αβ TCR dimer.

To examine the possibility that αβ TCR–related factors mediated the antigen-specific helper activity found in the supernatants of activated T_H cells, we tested the ability of $V_\beta 8$-specific F23.1 to bind to functional helper factors. Supernatants of activated T cell clones were incubated with Sepharose beads that had been coupled with purified F23.1 or with control proteins BSA or

Because the functional specificity of T_H supernatants closely resembled the specificity that is mediated by the αβ cell surface receptor on T_H cells, the presence of TCRαβ-related products in T_H supernatants was evaluated by biosynthetic labeling of T cell products with [^{35}S]methionine. Clones A-8, 8-5, and 10-5-17 were activated by incubation for 9 hours with immobilized anti-CD3 in the presence of [^{35}S]methionine. Both the cell pellets and the supernatants resulting from these cultures were then analyzed by immunoprecipitation and gel electrophoresis. Monoclonal antibody F23.1 (*9*), which is specific for determinants expressed on TCR $V_\beta 8$ gene products (*10*), precipitated from culture supernatants of clones A-8 and 10-5-17 (both of which are $F23.1^+$ and $V_\beta 8^+$), but not from clone 8-5 ($F23.1^-$), a product that migrated with an apparent molecular size of approximately 85 kD under nonreducing conditions (Fig. 2A) and 40 to 45 kD under reducing conditions (*11*). On reduced-nonreduced two-dimensional gel analysis, this product was shown to be a disulfide-linked ("off-diagonal") dimer (Fig. 2B) with characteristics identical to those of the cell surface αβ TCR (*1*). A parallel analysis of [^{35}S]methionine-labeled T cell lysates from the same clones showed identical results; spots isolated from clones 10-5-17 (Fig. 2B) and A-8 (*11*) were indistinguishable from material isolated from the supernatants of these clones.

monoclonal antibody to Ly6. Incubation with F23.1-Sepharose removed a substantial portion of the helper activity from the supernatant of the F23.1$^+$ clone A-8, as revealed by functional assay (Fig. 3B). In contrast, incubation with F23.1-Sepharose had no effect on the activity contained in the product of F23.1$^-$ clone 8.5 (Fig. 3A). BSA-Sepharose (Fig. 3) or anti-Ly6–Sepharose (*11*) had no effect on either clone product. An attempt was then made to recover helper factor activity by acid elution from F23.1-Sepharose or BSA-Sepharose. Previous experiments had demonstrated that the overall helper activity of T_H supernatants depended on the presence of lymphokines, including IL-4, in addition to any antigen-specific helper factor (*5*). The activity of the material eluted from F23.1 or control beads was therefore assayed in the presence of human recombinant IL-2 (rIL-2) and mouse recombinant IL-4 (rIL-4). As expected, no activity was recovered from F23.1-Sepharose or BSA-Sepharose that had been incubated with supernatant from the F23.1$^-$ clone 8-5 (*11*). In contrast, acid eluate from F23.1-Sepharose that had been incubated with supernatant from the F23.1$^+$ clone A-8 showed substantial helper acitivity. This activity was antigen-specific, supporting anti-TNP responses to TNP-KLH but not to TNP-OVA (Fig. 3C) and was MHC-restricted, helping responses by B10.A B cells but not by B10 B cells (Fig. 3D). Similar results were obtained with F23.1-Sepharose eluates of supernatants from the F23.1$^+$ clone 10-5-17 (*11*).

These findings demonstrate that the cell-free products of cloned T helper cells can subserve the function of intact T_H cells in the activation of B cell antibody responses through a cognate pathway, thus extending the findings of a number of investigators (*12–16*). Although the mechanism by which these factors work in activation of B cells is not known, it appears likely that the binding of factor to specific antigen presented in association with B cell Ia determinants provides a direct signal to the B cell, perhaps through cell surface Ia molecules as transducing structures. This signal, in synergy with those provided by lymphokines, may be capable of inducing B cell activation and differentiation to Ig secretion.

The T cell products we characterized are functionally antigen-specific and MHC-restricted, paralleling the—presumably TCR-mediated—recognition specificity of the T cells from which they are derived. Moreover, the specific helper factor produced by TCR $V_\beta 8$–expressing T_H cells can be bound by and eluted from a monoclonal antibody to $V_\beta 8$. The same T helper supernatants contain material that on reduced-nonreduced gel analysis is indistinguishable from the cell surface–derived αβ TCR. These functional and serologic similarities between the T_H cell αβ receptor and cell-free factor suggest that the factor represents a shed, secreted, or proteolytically cleaved form of the TCRαβ heterodimer. Similar suggestions have been made for T cell products that are active in cytotoxic T cell response (*17, 18*). Initial biochemical characterization of the antigen-specific helper factor analyzed in the present study demonstrated that both biologic helper activity and metabolically labeled αβ TCR material from T_H clone supernatants remain in the supernatant fraction after ultracentrifugation at 100,00*g* for 24 hours (*11*). Preliminary gel filtration analysis indicates that the biosynthetically labeled αβ dimer contained in clone supernatants has an apparent molecular size greater than 500 kD in its native state, suggesting that it exists in large complexes, potentially in association with other membrane components (*11*). If, as seems probable, the helper factor that has been isolated is a cell-free form of the αβ TCR, the ligand-binding properties as well as the effector functions mediated by this critical cell surface receptor can be studied directly.

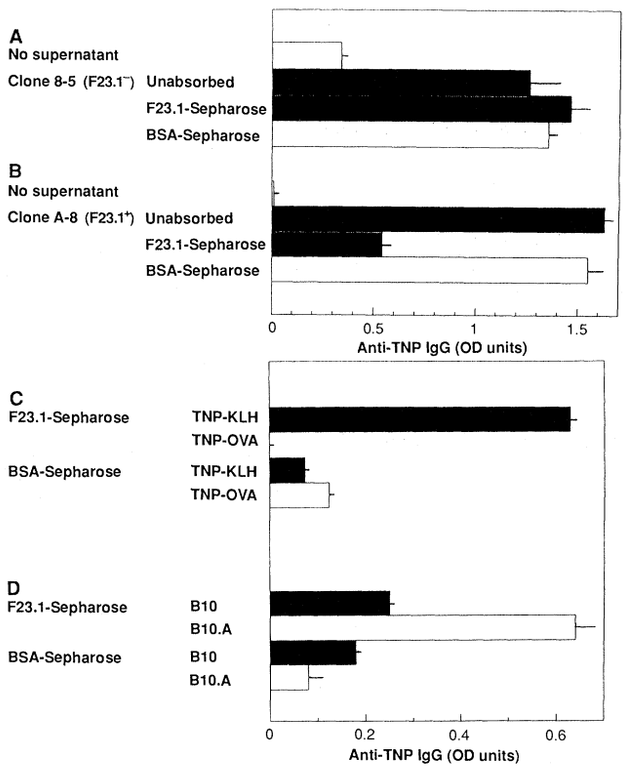

Fig. 3. Adsorption and elution of antigen-specific T helper factor activity with immobilized anti-TCR. T cell–depleted spleen cells (1.5×10^6) from TNP-KLH–primed B10 or B10.A mice were cultured in the presence of TNP-KLH, with or without supernatant from (**A**) F23.1$^-$ clone 8-5 or (**B**) F23.1$^+$ clone A-8. The supernatants tested for helper activity were unadsorbed or adsorbed on F23.1- or BSA-conjugated Sepharose 4B beads. Supernatant (2 ml) from anti-CD3–activated clones 8-5 or A-8 was incubated for 1 hour at 4°C with 0.1 ml of F23.1- or BSA-conjugated Sepharose 4B beads (1 mg of protein per milliliter of beads). After the incubation period the beads were centrifuged and the supernatants were tested for helper activity at a final dilution of 1:100 in the presence of rIL-2 (50 U/ml) and rIL-4 (100 U/ml). The antigen specificity and MHC restriction of T helper eluate from F23.1-Sepharose were assessed by (**C**) culturing T cell–depleted spleen cells (1.5×10^6) from TNP-KLH–primed B10.A mice in the presence of TNP-KLH or TNP-ovalbumin (OVA) with material that had been bound to and eluted from F23.1- or BSA-conjugated Sepharose 4B beads. (**D**) T cell–depleted spleen cells (1.5×10^6) from TNP-KLH–primed B10 or B10.A mice were cultured in the presence of TNP-KLH, with material eluted from F23.1- or BSA-conjugated Sepharose 4B beads. Supernatant (2 ml) from activated clone A-8 was incubated for 1 hour at 4°C with 0.1 ml of F23.1- or BSA-conjugated Sepharose 4B beads. At the end of the incubation period the beads were centrifuged and washed with phosphate-buffered saline; the adsorbed material was eluted by applying 1 ml of 0.1*M* acetic acid (*p*H 2.8) and collected into 0.1*M* tris buffered saline for immediate neutralization. Supernatants were tested for helper activity at a final dilution of 1:100 in the presence of rIL-2 (50 U/ml) and rIL-4 (100 U/ml). IgG antibody response was assayed by ELISA as described in Fig. 1. All data are presented as mean ± SEM of triplicate cultures.

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Degradation of Proteins with Acetylated Amino Termini by the Ubiquitin System

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A free NH_2-terminal group has been previously shown to be an obligatory signal for recognition and subsequent degradation of proteins in a partially fractionated and reconstituted ubiquitin proteolytic system. Naturally occurring proteins with acetylated NH_2-termini—most cellular proteins fall in this category—were not degraded by this system. Other studies have suggested that the identity of the NH_2-terminal residue is important in determining the metabolic stability of a protein in vivo (N-end rule). Whole reticulocyte lysate and antibodies directed against the ubiquitin-activating enzyme (E1) have now been used to show that such acetylated proteins are degraded in a ubiquitin-dependent mode. Although fractionation of lysate does not affect its proteolytic activity toward substrates with free NH_2-termini, it completely abolishes the activity toward the blocked substrates, indicating that an important component of the system was either removed or inactivated during fractionation. An NH_2-terminal "unblocking" activity that removes the blocking group, thus exposing a free NH_2-terminus for recognition according to the N-end rule, does not seem to participate in this pathway. Incubation of whole lysate with labeled histone H2A results in the formation of multiple ubiquitin conjugates. In contrast, the fractionated system is devoid of any significant conjugating activity. These results suggest that a novel conjugating enzyme (possibly a ubiquitin-protein ligase) may be responsible for the degradation of these acetylated proteins by recognizing structural features of the substrate that are downstream and distinct from the NH_2-terminal residue.

CONJUGATION OF UBIQUITIN TO certain proteins can trigger their degradation both in vitro and in vivo (*1*). A major question concerns the specificity of the system, that is, the identification of the structural features of a protein that render it susceptible to ubiquitin ligation. A free α-NH_2 group of some proteins is necessary for ligation of the proteins to ubiquitin and their subsequent degradation by crude reticulocyte fraction II [the high-salt eluate from reticulocyte lysate fractionated on a DEAE cellulose column (*2*)] supplemented with ubiquitin (*3*). Naturally occurring Nα-acetylated proteins are not degraded by this system (*3*). The identity of the NH_2-terminal residue affects the stability of a protein in vivo (*4*); genetically engineered derivatives of β-galactosidase had half-lives varying from a few minutes to many hours. Although it has recently been shown that the degradation signal includes a specific Lys residue that is proximal to the NH_2-terminal residue, the variation in the half-lives could be attributed entirely to the amino acid residue in the NH_2-terminal position (*5*).

We have shown that an Arg moiety must be transferred posttranslationally in a tRNA-dependent mode to acidic NH_2-termini (Asp or Glu) of proteins before their recognition by the ubiquitin system (*6*). A similar modification must also take place in proteins with an Asn, Gln, or Cys residue in this position (*7*). At least part of the selection of proteins for ubiquitin-dependent degradation is mediated by distinct recognition sites on ubiquitin-protein ligase (E3) for basic NH_2-termini (which also recognizes Arg-modified residues) and bulky hydrophobic NH_2-termini (*8*). It is also clear from these studies, however, that the NH_2-terminal residue, although an important recognition signal, is not the only one and most probably not the predominant structural feature. For example, a third site on E3 was classified that probably recognizes structural features of the proteins downstream from the NH_2-terminal residue (*8*). However, even the substrates recognized by this E3 had free NH_2-terminal residues; substrates with modified NH_2-termini were not recognized by this E3 and were not degraded by the DEAE-fractionated system (*2, 3, 8*). It is not known whether the different recognition sites reside within one E3 molecule or whether distinct E3 molecules recognize specific subsets of proteolytic substrates (*1, 8*).

Proteins with acetylated NH_2-termini constitute about 80% of the soluble cellular proteins in Ehrlich Ascites tumor cells (*9*). Apparently, most cellular proteins are degraded in an adenosine 5′-triphosphate (ATP)–dependent mode (*10*), and it is possible that the NH_2-terminal–blocked proteins could be degraded in the cell in an ATP-dependent, but ubiquitin-independent, mode (*11*). Another possibility is that these proteins can be degraded by the ubiquitin system; this possibility would imply that during the initial fractionation and separation of the system in vitro (*2*), a crucial factor necessary for their degradation is removed or inactivated. We have now tested these two hypotheses.

Table 1. Degradation of substrates with free and Nα-blocked NH_2-termini in crude reticulocyte fraction II and in reticulocyte lysate. BSA, lysozyme, α-crystallin (Sigma), histone H2A (Boehringer Mannheim), and actin (Worthington) were radioiodinated as described (*6*). In the case of α-crystallin, only the A chain was labeled, although both chains have blocked termini (*26*). Degradation was measured as described (*27*). Results represent a typical experiment. In five independent repeats with three different enzymatic preparations, the variation did not exceed 50% when degradation rates were below 10%, and 15% when degradation rates were above 10%. Each experiment was performed in duplicate. Variation among duplicates did not exceed 7%.

Substrate	Degradation (%)			
	Crude reticulocyte fraction II		Reticulocyte lysate	
	−ATP	+ATP	−ATP	+ATP
BSA	0	32	0	36
Lysozyme	7	29	5	31
Histone H2A	4	5	2	60
α-Crystallin	4	7	2	54
Actin	5	4	1	43

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